Molecular cytology of hypoxic cancer and stem cells: an epigenetic approach

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Abstract

Oxygen is an essential factor for life in many organisms. Oxygen concentrations vary widely across different human tissues, but in general, are much lower than the 21% oxygen in atmospheric air. In spite of this, most research continues to be based on the culture of cells in an air oxygen (21% O_2) environment. This ignores the mounting evidence of the role of physiological oxygen levels in the maintenance of survival, proliferation, stemness, genetic and epigenetic changes. The positive benefits of low oxygen tension on the maintenance of properties of several cells including embryonic and mesenchymal stem cells are well established while data describing negative impacts via genomic stability are few and conflicting. The role of cytosine modifications in cancer cells in response to hypoxia is poorly understood either *in vitro* or *in vivo*. In this study, we aimed to determine the role of low oxygen in the regulation of DNA methylation marks (5mC and 5hmC) and their associated genes including DNMT1/3A/3B/3L and TET1/2/3 in different cell lines including human embryonic stem cells, human mesenchymal stem cells and cancer cells. In addition to above, we also sought to corroborate upon and extend previous studies describing the effect of reduced oxygen on a range of cellular aspects including proliferation, metabolic activity, stemness and differentiation. To achieve these aims we cultured cells in either air oxygen, a fully defined 2% O_2 environment (Hypoxycool media, tri-gas workstation), or in a multiuser tri-gas incubator with handling in a standard biological safety cabinet.

Routine culture of stem cells in physiological normoxia enhanced the functional profile of stem cell populations including proliferation, metabolic activity and stemness. In contrast, culture of cancer cells in reduced oxygen caused significant decreases in growth profiles (vs. air oxygen). Quantitative RT-PCR and Western blots results of cells cultured in reduced oxygen revealed significant transcriptional translational downregulation of DNMT3B and TET1 vs. air oxygen (except for TET1 in cancer cell lines cultured in 2% O_2 where it

increased significantly), accompanied by significantly reduced in levels of 5mC and 5hmC (again except for 5hmC in cancer cell lines cultured in 2% O_2 where significant increases were noted). Noteworthy was that the downregulation in gene expression of DNMT3B was associated with an increase in its CpG promoter methylation. Importantly, these changes observation was associated with increases HIF2A, at protein levels in most cell types investigated. The role of physiological oxygen in these changes was confirmed by transitioning cancer cell lines between 2% O_2 and air oxygen and detailing the reversibility of both DNMT3B and DNMT3L expression at mRNA level and promoters CpG island methylation. Together these data suggest that the level of 5mC is reduced by HIF2A, via DNMT3B- mediated methylation.

In conclusions, our cells display oxygen-sensitive methylation patterns where *de novo* methylation is linked to oxygen culture and correlates directly with transcriptional and translational regulation of the *de novo* methylase DNMT3B. Delineating cancer and stem cell biology under niche-like culture conditions will ultimately enhance our understanding of mechanisms of action promoting improvements to both tumour-targeting medicines and regenerative medicine applications.

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Abbreviations

2%PG	2% O_2-Pre-gassed media in a 2% O_2 incubator
2%WKS	2% O_2-Pre-gassed media in a 2% O_2 Workstation
21%ST	21% O_2-Standard cell culture
5hmC	5-hydroxymethyl cytosine
5mC	5-methyl cytosine
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BMA	Bone marrow aspirate
BM-MSCs	Bone marrow-Mesenchymal stem cells
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU-F	Colony forming unit -fibroblast
CpG	Cytosine-guanine dinucleotide
DAP1	4,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5)-methyltransferase
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNMT3B	DNA (cytosine-5)-methyltransferase 3B
DNMT3L	DNA (cytosine-5)-methyltransferase 3-like
dNTP	Deoxynucleotide triphosphates
DPBS	Dulbecco's Phosphate Buffered Saline
E8M	Essential 8 Medium
EDTA	Ethylene diamine tetraacetic acid
ESCs	Embryonic stem cells
FBS	Fatal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
HDAC	Histone deacetylates histone methyl transferases
hESCs	Human embryonic stem cells
HIFs	Hypoxia inducible factors
HIF1A	Hypoxia inducible factor 1-alpha
HIF2A	Hypoxia inducible factor 2-alpha

HIF-1β	Hypoxia inducible factor 1-beta
hiPSCs	Human induced pluripotent stem cells
HLA-DR	Human Leukocyte Antigen-antigen D Related
hPSCs	Human pluripotent stem cell
HMAs	Hypoxia mimetic agent
HMT	Histone methyl transferases
HRE	Hypoxia response elements
HRP	Horse raddish peroxidase
IBMX	3-isobutyl-1-methylxanthine
ICM	Inner cell mass
IgG	Immunoglobulin G
KO-DMEM	Knockout-Dulbecco's Modified Eagle's Medium
MBD	Methyl CpG binding domain
MeCP2	Methyl CpG binding protein 2
mESC	Mouse embryonic stem cell
MLL	Acute myeloid leukemia
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NANOG	Homeobox Transcription Factor
NEAA	Non-Essential amino acid
OCT-4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PHD	Prolyl-hydroxylases
p-VHL	Von Hipple-Lindau tumour suppressor
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SOX-2	SRA (sex determining region)-box
SSEA	Stage specific embryonic antigen
TAE	Tris acetate Ethylene diamine tetra acetic acid
TET	Ten-eleven translocation methylcytosine dioxygenase
TFBS	Transcription factor binding sites
TSS	Transcription start sites
VEGF	Vascular endothelial growth factor

Presentations

- Rakad M Kh AL-Jumaily, Wen-Wu Li, Nickolas R Forsyth. Molecular cytology of hypoxic cancer and stem cells; an epigenetic approach. ^{10}th Annual ISTM PG Symposium. 2015.
- Rakad M Kh AL-Jumaily, Wen-Wu Li, Nickolas R Forsyth. Low oxygen culture modifies the epigenetic state of pluripotent stem cells. ^{11}th Annual ISTM PG Symposium. 2016.
- Rakad M Kh AL-Jumaily, Wen-Wu Li, Nickolas R Forsyth. Hypoxia modifies the epigenetic state of induced pluripotent stem cells. MSCA/Manchester. 2016.
- Participate in Chapter 9 of (Cell Culture Techniques). 2016.
- Rakad M Kh AL-Jumaily, Wen-Wu Li, Nickolas R Forsyth. Physiological normoxic culturing caused DNA hypomethylation in human bone marrow-derived mesenchymal stem cells, HIF2A dependent. TCES conference/ Manchester. 2017.
- Nicholas R Forsyth, Mark Kitchen, Rakad M K Al-Jumaily. Oxygen-driven Regulation of DNMT3B Defines Methylation Status in Human Mesenchymal Stem Cells. ^{5}th TERMIS - World Congress-2018. Kyoto, Japan.

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Keele University

Chapter 1: Introduction

1-Introduction

Epigenetics research has recently been increases remarkably in an attempt to understand the mechanisms of gene expression, cellular development and disease. Epigenetics describe the heritable changes in gene function that occur without changes in the DNA sequence (Zhang et al., 2010; D'Urso and Brickner, 2014). The molecular basis of heritable epigenetics has been examined in a variety of organisms and there are many mechanisms by which epigenetics can control the process of gene expression, but generally these include: DNA methylation (chemical alteration of DNA); modifying the histone proteins related to DNA (histone modication) and chromatin remodelling. There is a complex interplay between these operations and they all act to regulate the activity of gene expression (Bird, 2007; Bossdorf et al., 2007; Gal-Yam et al., 2008).

In parallel to the study of epigenetics progress has been made in the study of stem cells. It is the characteristics of these cells that provide hope and promise for the treatment of many diseases and disorders including cancer, Alzheimer's disease, Parkinson's disease, Cardiac regeneration, and diabetes amongst other diseases (Sager et al., 2007; Jung et al., 2012). In addition, stem cell research has played a significant role in promoting understanding of the basics of differentiation and growth, as well as the evolution of diseases such as cancer (Rubio et al., 2005). Stem cells are characterized by three key properties which makes them distinct from other cells. Firstly, stem cells have the capacity to divide and self-renew, unlike other cells in the body; secondly, stem cells are non–specialized cells; thirdly, stem cells can differentiate and turn into multiple types of specialized cells found within the body, such as blood cells, bone cells, etc. under physiological conditions and in response to activation of specific families of genes. Differentiation capacity is ultimately dependent on the potency and variety of stem cell under investigation. Researchers predominantly utilize two main

sorts of stem cells for research purposes: embryonic or pluripotent stem cells and adult stem cells such as mesenchymal stem cells (Polok and Bishop, 2006).

The relationship between stem cells and their niche and the significant role that the niche plays in maintenance, self-renewal and differentiation is currently under examination. The niche itself refers to the unique local microenvironment where an individual population of stem cells reside. Stem cells react with their niche through adhesion molecules and subsequent biochemical signalling translated into transcriptional machinery that preserves the characteristics of stem cells. Greater understanding of the nature of stem cells and their niches are expected to offer alternative routes in the handling of different diseases such as malignant neoplastic disease (Iwasaki and Suda, 2009). It is also clear that oxygen tension is involved in the regulation of stem cell behaviour and that it is an essential part of the stem cell microenvironment (Ma et al., 2009). Recent investigations have demonstrated that hypoxic niches can play a part in maintaining the undifferentiated state of several stem cells, including embryonic, mesenchymal and cancer stem cells and as well as having strong effects on proliferation and differentiation (Mohyeldein et al., 2010).

Despite improvements in diagnosis and treatment of cancer, it is still one of the major causes of death in the world alongside heart diseases. Cancer is a disease characterized by uncontrolled cellular proliferation and differentiation (Parkin et al., 1999; Alghamdi and Khorshid, 2012). It is well-recognized that the development and progression of many cancers are influenced by the interactions between cancer cells and their local tumour microenvironment. Clinical investigations have indicated that hypoxia is associated with several tumour types, especially solid cancers (De Veirman et al., 1014). On the other hand, it has been shown that hypoxia is responsible for reducing DNA repair processes and, consequently, increasing mutagenesis (Vaupel, 2004).

Recently, it has been reported that cancer appears to have both epigenetic and genetic basis (Sadikovic et al., 2008). In addition, the role of epigenetic mechanisms and their responses to carcinogens has been explored. Recent research has indicated that cancer cells display similar epigenetic modifications to those associated with the differentiation of a stem cell (Ortiz-Sa'nchez, 2014). Moreover, there is increasing evidence that hypoxia plays an essential part in gene regulation (Weston et al., 2010). Therefore, better understanding of epigenetics changes could trigger new approaches, to understanding how the local microenvironment can modify cells to trigger disease without initiating mutations as a key primary process of disease development.

The figures pathways presented in this thesis are generic, exist in most cell types and all exceptions was listed.

1.1 Epigenetics

In 1942, the term epigenetics was used for the first time by Conrad Waddington to describe the interactions between genotype and phenotype during development. He had hypothesized that non–genetic factors participate in the process of development of embryogenesis (Waddington, 1942; Altun et al., 2010). Epigenetics was also used to answer questions that cannot be explained by genetics alone, such as why identical twins have different types of diseases, although they have the same DNA sequence? Also, why do all cells in the body have the same genetic information, but tissue and organs have different functions? (Jorde and Wooding, 2004; Altun et al., 2010). The term epigenetics has been outlined in numerous ways over the years. At this time, epigenetics is defined as “the heritable changes in phenotype or gene expression not caused by the alteration in the underlying DNA sequence” (Probst et al., 2009). The basis of molecular epigenetic modifications has been explored in many organisms (Bird, 2007). However, there are various mechanisms by which epigenetics

can regulate gene expression; but in general, they are divided into either chemical modulation of DNA (DNA methylation) and histone modifications (Allen et al., 2009). These mechanisms play an essential role as modifications can have significant impact on chromatin structure and transcriptional activity of genes. In contrast to genetic aberrations, epigenetic changes are a reversible phenomenon (Boultwood and Wainscoat, 2007). Furthermore, these mechanisms play a fundamental role in different biological processes such as in normal mammalian development, embryogenesis, cellular differentiation, and cancer biology (Dueñas-González, 2005; Wegman-Ostoisky et al., 2007). Principally, these alterations can work alone, or in combination, to generate conformational modifications (remodelling) of chromatin which influences its accessibility to transcriptional machinery, and which thereby regulates transcriptional capability (Narlikar et al., 2002).

1.1.1 DNA methylation (5mC)

The first described covalent modification of DNA and the most extensively studied epigenetic modification to date is DNA methylation (Lechner et al., 2010; Sharam et al., 2010; Dawson and Kouzarides, 2012). In mammalian cells, DNA methylation (5mC) refers to the covalent modification of the cytosine base that primarily occurs in the context of a CpG dinucleotide within the DNA sequence (i.e. a cytosine immediately followed by a guanine) through the addition of a methyl group at 5-carbon position on the cytosine nucleotide (Issa, 2012). In general, the epigenetic mark (5mC) can alter the regulatory function of these regions without changing the Watson-Crick base pairing of cytosine (Schübeler, 2015). This process can alter the biophysical characteristics of DNA and cause two effects; blockage of recognition sites of DNA by DNA-binding proteins (MBD) as well as allowing other proteins to bind leading to activation or inhibition of the target gene (Damelin and Bestor, 2007; Prokhortchouk and De fossez, 2008). However, the distribution of CpG dinucleotides in mammals is not uniform within the genome instead being

concentrated within small stretches of DNA known as CpG islands. Remarkably, many CpG islands are found associated with gene promoter regions such as housekeeping genes and tissue-specific genes as well as developmentally regulated genes. Furthermore, in contrast to non-island associated CpG sites, which are almost always methylated (approximately 70%), the CpG sites within promoter associated CpG islands are usually methylation free (Suzuki and Bird, 2008; Zhu et al., 2008). The methylation within CpG islands regions often serves a key function in a variety of cellular processes including transcriptional silencing, genomic imprinting and X-chromosome inactivation (Sadikovic et al., 2008; Sharam et al., 2010; Kanwal and Gupta, 2012). Mohn *et al* reported that methylation of CpG islands delivers stable silencing of relevant genes which is necessary for normal development and cell differentiation (Mohn et al., 2008). An inability to maintain DNA methylation, thereby causing aberrant methylation patterns resulting in under-expression and overexpression of certain proteins is a hallmark phenotype of a range of diverse diseases including cancer (Sharam et al., 2010; Tellez-plaza et al., 2014).

1.1.2 5-Hydroxymethylcytosine (5hmC)

A further mechanism thought to be involved in the regulation of DNA methylation patterns is the active demethylation of 5mC to 5‑hydroxymethylcytosine (5hmC) by the ten-eleven translocation family of deoxygenases (TETs) (Williams et al., 2011; Putiri et al., 2014). Historically, 5hmC was first reported in DNA mammals in the early 1970s but its function as an epigenetics mark was not discovered until 2009, when researchers noticed a significant quantity of 5hmC in Purkinje neurons and brain tissue (Penn et al., 1972; Kriaucionis and Heintz, 2009). Recently, several potential roles of 5-hydroxymethylcytosine (5hmC) in different organs have been explored (Tellez-Plaza et al, 2014, Fong et al., 2018). In general, 5hmC is formed by oxidation of the methyl group in 5mC, by either TET1, TET2 or TET3

(Tan and Shi, 2012) as shown in **Figure 1.1**. However, following on from 5hmC detection in embryonic stem cells researchers have suspected that 5hmC may help determine development fate and be associated with the maintenance of transcriptional regulation and differentiation in ES cells as well as regulation of gene expression in the central nervous system (Li et al., 2013). Genome wide identification studies of ESCs have located high concentrations of 5hmC at transcription start sites (TSSs) of CpG-rich promoters (Williams et al., 2011; Ecsedi et al., 2018). Furthermore, global decreases in 5hmC level have been noted in many neoplasia including colon, prostate and breast through changes in the level of TETs expression (Ko et al., 2010; Haffner et al., 2011; Uribe-Lewis et al., 2015). Reports have now shown that the location of DNA methylation within a gene and its type are essential in determining its function. Importantly, it has observed that 5mC and 5hmC always co-exist, but they have different regulatory functions at gene promoters (Williams et al., 2011; Xu et al., 2011). For example, 5mC is often associated with repression transcription whereas 5hmC is involved in increased gene expression (Mariani et al., 2014). The role of the "sixth base" in the epigenetic control of gene regulation and disease development is under investigation (Chen et al., 2012; Ecsedi et al., 2018). This study will refer to 5mC and 5hmC collectively as DNA methylation and individually as cytosine methylation (5mC) and cytosine hydroxymethylation (5hmC).

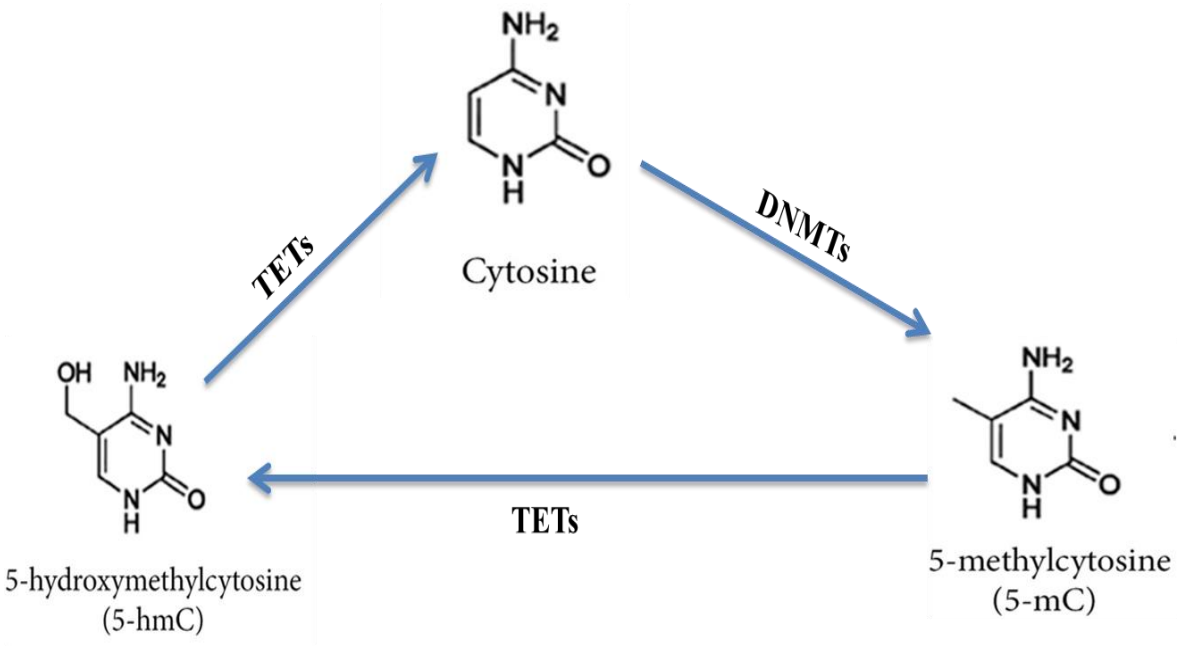


Figure 1.1 Chemical structures of 5-methylcytosine (5mC) and its oxidation products 5-hydroxymethylcytosine (5hmC). DNA methylation occurs at cytosine bases when a methyl group is added at the 5' position on the pyrimidine ring by a DNA methyltransferase (DNMTs). Demethylation of 5mC to 5‑hydroxymethylcytosine (5hmC) occurs by ten-eleven translocation family of deoxygenases (TETs).

1.1.3 Regulation of DNA methylation

Three catalytically active members of methyltransferase enzymes (DNMTs) are directly involved in establishing and maintaining the cytosine methylation patterns in mammals including DNMT1, DNMT3A and DNMT3B (Cheng and Blumenthal, 2008). Functionally, these enzymes have been further categorised into two different classes as shown in **Figure 1.2**. DNMT1, which is involved in the maintenance of heritable epigenetic marks during cell division, and DNMT3A and DNMT3B, which work in co-ordination with DNMT1 and are responsible for *de novo* methylation, typically during early development (Gopalakrishnan et al., 2008). DNMT1 is mainly produced during the S phase of the cell cycle and possesses a strong affinity for the hemimethylated DNA genome (Robertson et al, 2000; Dhe-Paganon et al., 2011). The importance of normal formation and maintenance of DNA methylation

patterns during growth and development have been confirmed in several gene targeting experiments (Sadikovic et al., 2008). Mouse homozygous mutants deficient in DNMT1 expressed a lethal phenotype, resulting in stunted development, and gross morphological abnormalities where consequentially the embryos did not survive past mid-gestation (Li et al., 1992).

DNMT3A and DNMT3B are both highly expressed during embryonic development and down-regulated post-differentiation (Turek-Plewa and Jagodzinski, 2005; Athanasiadou et al., 2010). Further evidence of the importance of DNMT3A and DNMT3B in *de novo* methylation was observed in mutation studies in embryonic stem cells (ESCs). Double mutant ESCs [Dnmt3A-/-, Dnmt3B-/-] showed a complete lack of d*e novo* methylation (Okano et al., 1999). In addition, inactivation of both DNMT3 enzymes in mice results in embryonic lethality (Jones and Baylin, 2002). In addition to these two groups of enzymes, two further kinds of DNMT like proteins, particularly, DNMT2 and DNMT3L are also associated with DNA methylation. DNMT3L is homologous to DNMT3A and DNMT3B and it has been documented that DNMT3L specifically recognizes non-methylation histone and acts as a cofactor involved in the methylation of DNA by both enzymes (DNMT3A and DNMT3B) (Wienholz et al., 2010). Furthermore, a recent study has shown that DNMT3L takes part in maternal methylation imprinting and that the absence of DNMT3L in mice leads to defects in methylation of Oocytes and Spermatogenesis (Suetake et al., 2004).

On the other hand, it has been reported that 5hmC is involved in the regulation of DNA methylation by acting as a guardian of CpG islands (maintaining them as DNA methylation free) (Williams et al., 2012). The fact that 5hmC is enriched at promoter regions and transcription start sites (TSSs) suggests that 5hmC may be derived from pre-existing 5mC being converted to 5hmC specifically at CpG islands (Williams et al., 2012). Le *et al* found in bacteria that 5hmC can be converted back to 5mC through removal of a formaldehyde

group from 5hmC by the DNMT enzymes (Le et al., 2011). However, consistent with the role of TETs in converting 5mC into 5hmC *in vivo*, TET1 knockout mESCs have decreased levels of 5hmC accompanied by a slight increase in global level of 5mC (Dawlaty et al, 2011).

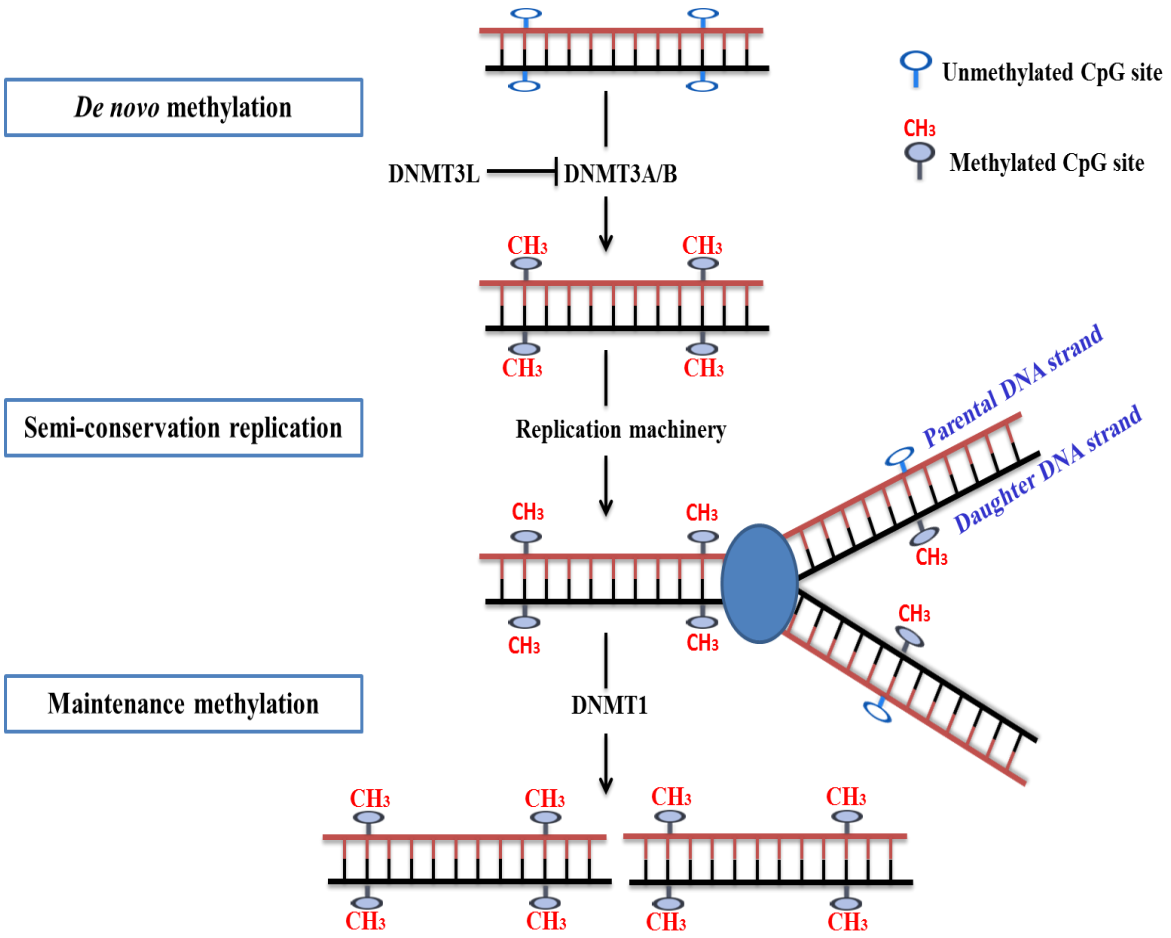


Figure 1.2 *De novo* and maintenance DNA methylation during replication in mammalian cells. Maintenance methylation activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. *De novo* methylation is catalysed by DNMT3A/B during semi-conservative replication, the resultant daughter DNA strands are hemi-methylated. DNMT1 is involved in the maintenance of heritable epigenetic marks during cell division and possesses a strong affinity for hemimethylated DNA genome. The actions of the de novo methyltransferases are regulated by DNMT3-like protein (DNMT3L) which can stimulate or repress the activity of these enzymes.

1.1.4 DNA methylation regulates gene expression

DNA methylation plays an essential role in the repression and promotion of gene expression. The mechanism by which methylation represses gene expression is yet to be established but it is suggested that gene promoters are unmethylated to allow transcription factors to bind to their binding site (Chen et al., 2011). In line with this idea, Philips *et al* reported that the levels of methylation vary near the transcription sites depending on cell type. In addition, this change in extensive methylation patterns at gene promoters correlates with reductions in gene expression (Philips, 2008). However, there are two potential mechanisms for the regulation of gene expression by DNA methylation as shown in **Figure 1.3**. Firstly, some transcription factors are unable to bind to their binding site if it contains methylated cytosine. Secondly, induced gene silencing through recruitment of methyl CpG binding domain (MBD) proteins which interact with a cytosine methyl group leading to alteration of the chromatin activity via complex development with other particles such as histone modification enzymes including histone deacetylates (HDACs) and histone methyl transferases (HMTs) (Fuks et al., 2001; Fatemi and Wade, 2006; Klose and Bird, 2006). MBDs constitute a family of five methyl-binding proteins which are known as epigenetic methyl signature readers which include MeCP2, MBD1, MBD2, MBD3 and MBD4 (Fatemi and Wade, 2006). In addition, it has been reported that most of the MBD family members associate with large protein complexes containing nucleosome remodelling activity and histone deacetylation activity (HDACs) that are involved in the process of transcription silencing through modification of chromatin components (Fatemi and Wade, 2006). On the other hand, little information is known about the role of 5hmC as a gene expression regulator, but it has reported that 5hmC is involved in regulation of gene expression through altering chromatin structure or recruitment or displacement of other DNA binding proteins that affect transcription (Fong et al., 2008). Zhang *et al* reported that 5hmC is involved in gene

regulation through inhibition of the binding of methyl CpG binding proteins 2 (MBP2) at CpG sites (Zhang et al., 2012). In line with this idea, TETs may be involved in gene regulation through adjusting DNA methylation levels at promoter regions and this finding is supported by fact that both TETs and 5hmC localise to TSSs in mESCs (Williams et al., 2011). However, Wu *et al* reported that 5hmC at promoter regions is associated with gene repression while within the gene was associated with active gene transcription (Wu et al., 2011).

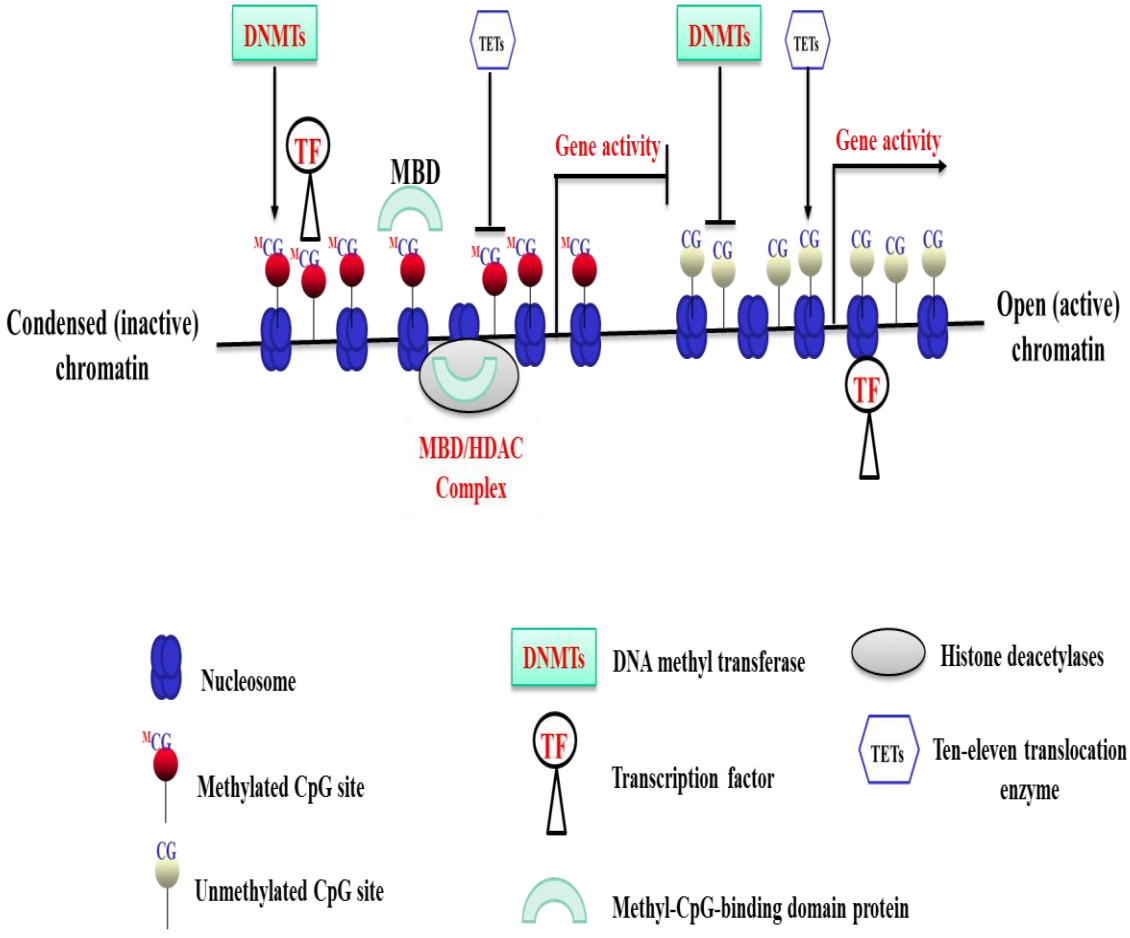


Figure 1.3 Regulation of gene expression by DNA methylation. DNA methylation can alter the biophysical characteristics of DNA and cause two effects; blockage of recognition site of DNA by DNA-binding proteins as well as allows other proteins to bind leading to activation or inhibition of the target gene in these regions.

1.2 Stem cells

Stem cells are defined as undifferentiated or unspecialised cells that can differentiate and generate more specialised cells. Stem cells can be divided into two main types: embryonic stem cells from the embryo and adult stem cells from adult tissue. Stem cells have the capacity to self-renew and differentiate into one or more types of specialized cell in the body (Polok and Bishop, 2006). Stem cells are classified according to their capacity for differentiation as shown in **Figure 1.4**. Examples are:

- Totipotent stem cells are found only in early progeny of the zygote (1-3 days). These cells have ability to differentiate into all types of specialized cells found within the body and the placenta and subsequently develop into a complete organism (Mitalipov and Wolf, 2009).
- Pluripotent stem cells are derived from the undifferentiated inner cell mass (ICM) of the blastocyst (5-14 days). These cells can differentiate into cell types representative of the three germ layers (endoderm, mesoderm and ectoderm) such as, embryonic stem cells (Mitalipov and Wolf, 2009).
- Multipotent stem cells. These cells have the basic characteristics of all stem cells. These cells can differentiate into multiple cell types in a single germ layer. An example is the multipotent blood stem cell (Hematopoietic) which can differentiate into roughly 11 cell types found within blood such as red blood cells, white blood cells or platelets (Bianco, 2011).

In addition to those above unipotent stem cells represent another group of stem cell with the capacity to differentiate into only one specific cell type (Nii et al., 2014).

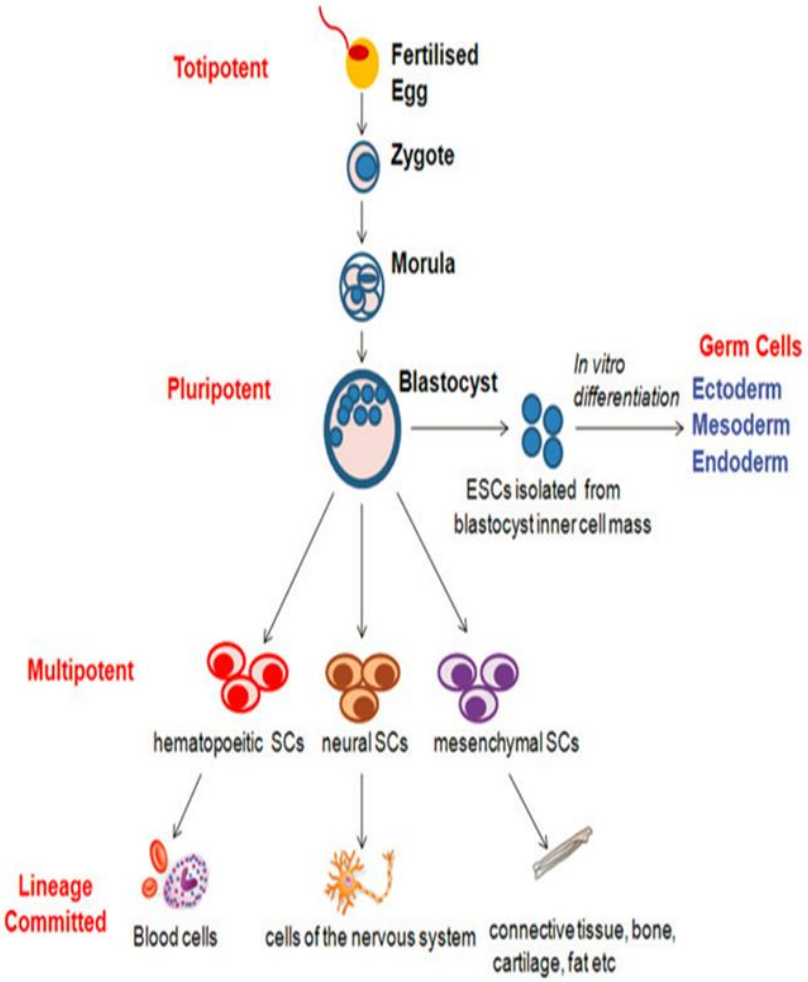


Figure 1.4 Schematic diagram illustrating the stem cell hierarchy. Embryonic stem cells (ESCs) are pluripotent and have the capacity to differentiate into cells of all three dermal layers, that is, endoderm, mesoderm and ectoderm. Adult stem cells are multipotent, and have a more restricted ability to differentiate, being committed to a specific lineage (Hayes et al., 2012).

1.2.1 Human pluripotent stem cells

1.2.1.1 Human embryonic stem cells

The therapeutic potential of stem cells holds great promise in treating many human diseases due to their unique properties. Human embryonic stem cells (hESCs) are derived from the undifferentiated inner cell mass (ICM) of the blastocyst (Watt and Driskell, 2010). ESCs are pluripotent and are characterized by their potential to replenish themselves (self-renew) and

their ability to differentiate into cell types representative of the three germ layers (endoderm, mesoderm and ectoderm) and form any of the over 200 different cell types found in the body (De Wert and Mummery, 2003; Watt and Driskell, 2010). Thus, they have been considered to have brought great hope to the field of regenerative medicine therapy, tissue transplantation and drug discovery (Ho et al., 2012; Nii et al., 2014). In 1998, the first human embryonic stem cell lines were developed successfully in plastic laboratory culture dishes by Thomson and colleagues (Thomson et al., 1998). Study of undifferentiated hESCs with electron microscopy has shown that hESCs have a high ratio of nucleus to cytoplasm, prominent nucleoli, indistinct cell membranes and free ribosomes (Oh et al, 2005). In addition, hESCs are characterized by the expression of a set of cell surface markers such as SSEA-4, SSEA-3, TRA-1-60 as well as TRA1-80 and to lack expression of a lactoseries oligosaccharide antigen, SSEA-1 that is expressed upon differentiation, and expression of transcription factor OCT-4 (Vazin and Freed, 2010).

Maintenance of pluripotency and self-renewal is an important feature of hESCs and understanding the mechanisms involved in hESCs differentiation *in vitro* is essential for further understanding of the development of functional cells. The OCT-4, SOX-2 and NANOG transcription factors are important regulators that govern the self-renewal of hESCs through activation or repression of gene expression (Boyer et al., 2005; Galvagni and Neri, 2015). During mammalian development, OCT-4 is an essential regulatory molecule that is implicated in the initial cell fate decision (Loh et al., 2006). Indeed, overexpression of OCT-4 in hESCs pushes differentiation towards primitive endoderm and mesoderm whereas disruption of OCT-4 leads to dedifferentiation toward the trophectoderm (Matin et al., 2004). In addition, it is reported that OCT-4 interacts with SOX-2 and both are involved in gene activation and repression *in vivo*. NANOG is essential to block differentiation and can

preserve the pluripotent state of ESCs during embryonic development (Boyer et al., 2005, Saunders et al., 2013).

1.2.1.2 Induced pluripotent stem cell

Induced pluripotent stem cells (iPSCs) have been added to the field of stem cells with similar embryonic stem cells characteristics including morphology, proliferation ability as well as the expression of ESCs specific cell surface antigens (SSEA-3, SSEA-4, TRA-1-60 and TRA1-80) (Mitalipov and Wolf, 2009). Induced pluripotent stem cells are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state via transduction of four defined factors (OCT-4, SOX-2, Klf4 and c-Myc) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Mouse iPSCs were first reported in 2006, and human iPSCs were first reported in late 2007 (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Human iPSCs express the endogenous genes (OCT-4, SOX-2 and NANOG) and can generate cells characteristic of all three germ layers (Salci et al., 2015).

1.2.2 Adult stem cells

Adult stem cells (ASCs), which also includes fetal and somatic stem cells, are found in tissues such as bone marrow, blood, liver, skin and muscle amongst other tissues and are present as undifferentiated cells amongst other differentiated cells (Fujimaki et al., 2013). Adult stem cells within tissues have the ability to support repair and differentiate into other representative specialized cell types (Kondo, 2010). The typical role of an adult stem cell is to maintain and replenish their tissue of origin throughout an individual's life (Fujimaki et al., 2013).

In general, adult stem cells can be classified depending on (1) The ability to differentiate into different cell types where adult stem cells can have two kinds of cell potency including

multipotent stem cells such as, human mesenchymal stem cells (hMSCs) and unipotent stem cells such as muscle stem cells (Meirelles, 2009). (2) The germ layer they come from, for example, endoderm layer makes up pancreas, lung, intestine and liver while ectoderm layer gives rise the skin, nervous system, hair and mammary glands. On the other hand, muscle, mesenchymal stem cells and circulatory system generated from mesoderm layer (Haasters et al., 2009).

1.2.2.1 Mesenchymal stem cells

Bone marrow (BM) is the most common source for isolation of mesenchymal stem cells, but other tissues such as adipose tissue, compact bone, liver, intestinal tract, kidney, and umbilical cord blood have also been utilised as a source for isolation of mesenchymal stem cells (Gao et al., 2014). Mesenchymal stem cells (MSCs) are a specific rare population of adherent cells that constitute about 1 in 10,000 of bone marrow mononucleated cells. These cells are characterized by the ability to produce single cell colonies, and their capacity to adhere to tissue culture plastic. These colonies are termed the CFU-F (Colony Forming Unit–fibroblast) (Wang and Wagers, 2011). The first descriptions of human mesenchymal stem cells (hMSCs) were discovered by Friedenstein and colleagues in the early 1970s as a non-hematopoietic stem cell. It was established that hMSCs were able to differentiate into a range of lineages including osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells) (Friedenstein et al., 1968; Karystinou et al., 2009). Furthermore, several recent studies have observed that hMSCs can differentiate into other lineages such as myocytes and neurons (non-mesenchymal lineages) (Bossolasco et al., 2005). In addition to multipotency, one of the main properties of MSCs is their inability to self-renew and that the maintenance of the non-differentiated state is lost over several cell divisions (Sethe et al., 2006). *In vitro*, BM-derived MSCs can be cultured easily and can be expanded to relatively high numbers for use in different applications (Bernardo et al., 2007). In general, hMSCs have three

primary functions linked to their potential therapeutic use including tissue repair via molecule secretion, tissue replacement through multipotent properties, and their immunomodulatory functions (Devine et al., 2003; Ryan et al., 2005). By contrast, information about hMSCs *in vivo* is very poor for two reasons, rarity in different tissues as well as the difficulty of isolating a large quantity and high purity of hMSCs (Boguest et al., 2005). However, MSCs usually exist in a very low concentration within a given tissue. In order to distinguish them from other cell populations, it is possible to exploit the cell surface markers which are unique for each cell type. These are proteins molecules, also known as "receptors" that coat the surface of all cells, and are able to bind to other cells, surfaces or proteins. CD (cluster of differentiation) markers are commonly used to identify stem cell types in bone marrow, but additionally a number of antibody-binding receptors, antigens, can also be used (Chippendale et al., 2011). However, the International Society for Cellular Therapy has defined minimal guidelines for MSCs characterisation including; plastic adherence, multi-lineage differentiation into fat, bone, and cartilage, together with a surface expression of stem cell markers (CD73, CD90, and CD105), with lacking expression of haematopoietic markers (CD34, CD45, CD11a, CD19 or CD79a, CD14 or CD11b and histocompatibility locus antigen (HLA)-DR) (Dominici et al., 2006). It must also be considered that many different cell types may have a number of markers in common, so in order to isolate a specific stem cell population contained within the highly heterogeneous bone marrow, or to further separate the sub-populations of the adherent fraction, a combination of different markers must be used (Chippendale et al., 2011).

1.2.3 The role of DNA methylation in stem cell regulation

DNA methylation plays a major role in the regulation of stem cell differentiation and is essential for maintenance of stem cell characteristics (Huang et al., 2013). hESCs have been shown to have unique DNA methylation marks when compared to DNA methylation status

of both differentiated cells and cancer cells (Bibikova et al., 2006; Altun et al., 2010). However, high expression levels of the *de novo* methyltransferase enzymes (DNMT3A and DNMT3B) occur during development especially in undifferentiated cells which reduce during cellular differentiation (Li et al., 2007; Totonchi et al., 2017). In addition, the pluripotency-associated genes such as OCT-4 and NANOG become methylated (silenced) to allow stem cell to differentiate into specific lineages (Gopalakrishnan et al., 2008; Pelosi et al., 2011). Moreover, as differentiation continue, the global DNA methylation decreases but the fundamental methylation profile is maintained by DNMT1 (Ludwig et al., 2014). The significance of methylation on the genes that associate with pluripotency is dependent on the stages of embryogenesis and on specific cell types. For example, promoter regions of DNA can be hypomethylated in mouse ES cells but are hypermethylated in trophoblast ES cells (Gopalakrishnan et al., 2008). Furthermore, Ho *et al* found that some genes were unmethylated in ESCs with no transcriptional, but that these genes could be supressed by DNA methylation during differentiation (He et al., 2018). Changes in DNA methylation are important for stem cell differentiation as methylated DNA alters the interaction between transcription factors and their binding sites (TFBSs) on DNA (Chen et al., 2011) or by formation of complex with other molecules such as histone modification enzymes that alter chromatin activity (Hutnick et al., 2009).

However, knockout of DNMT leads to loss ESCs the ability to differentiate while retaining the ability to replenish themselves (self-renewal). These results suggested that DNMTs play a prominent epigenetic role in pluripotency (Okano et el., 1999; Hu and Rosenfeld, 2012). In addition, another DNA methylation mechanism is involved in regulation of hESCs pluripotency; TETs enzymes (Moore et al., 2012). It has reported that global 5hmC levels significantly changed during lineage commitment of pluripotency stem cells into neural cells (Kim et al., 2014). On the other hand, knockout of TET1 in mESCs results in reduced levels

of 5hmC and an associated slight increase in global 5mC level (Dawlaty et al., 2011). Recently, Samadian *et al* found that the expression of TET1 was significantly upregulated during embryonic stem cells (ESC) derivation. Moreover, they also demonstrated that maintaining DNA methylation at low levels was essential to establish the ground-state of pluripotency during the early days of ESC derivation (Samadian et al., 2018).

1.3 Cancer: Genetics versus Epigenetics

Despite significant progresses in diagnostics and treatment methods and ongoing basic research of cancer disease, it is still the major cause of death in the worldwide alongside cardiac diseases (Parkin et al., 1999; Han et al., 2017). Traditionally cancer has been viewed as a disease characterised by the accumulation of genetic mutations, resulting in a multistep process of tumour pathogenesis (Hanahan and Weinberg, 2011; You and Jones, 2012). Loss or gain of function mutations of tumour suppressor genes and/or oncogenes respectively contribute to the development of a multitude of cancer hallmarks, including sustained proliferation, resistance to cell death and the ability to metastasise (Bertone et al., 2004; Cheng et al., 2005; Hanahan and Weinberg, 2011). Nonetheless, genetic alterations in cancer alone does not seem to present a complete explanation of the complexities of carcinogenesis and the acquisition of tumour heterogeneity (Sadikovic et al., 2008; Kanwal and Gupta, 2012). Recently, it has been reported that cancer appears to have both an epigenetic and genetic basis, which is thought to interact to potentiate tumourigenesis (Baylin and Herman, 2000; Baylin and Jones, 2011). Whilst the genetic aetiology of cancer has been well researched, the role of epigenetic alterations in response to changes within the tumour microenvironment is poorly understood (Baylin and Jones, 2011). Notably, targeting epigenetic mechanisms may present a significant potential for clinical translation, as despite the heritability of epigenetic marks, the ability to reverse these changes presents new opportunities for therapeutic strategies, highlighting the importance of understanding cancer

epigenetics (Jacqueline and Wainscoat, 2007; Kanwal and Gupta, 2012; Kagohara et al., 2018).

1.3.1 Role of DNA methylation in cancer

Tumour initiation and progression have been known to be aided by the DNA methylation of the CpG islands of the promoter region of tumour suppressor gene which is the best studied epigenetic modification in cancer, and this is present in about 70% of all mammalian promoters (Dawson and Kouzarides, 2012). However, during tumour initiation and progression, cancer cells are marked by major disruptions in their DNA methylation profiles, displaying a complete reversal to the normal methylation patterns observed in healthy cells (Sadikovic et al., 2008; Sharma et al.,2010). This includes hypermethylation of CpG islands in gene promoter regions and global hypomethylation, resulting in alterations of key genes and downstream signalling pathways (Sadikovic et al., 2008; Sharma et al.,2010; You and Jones, 2012).

In cancer, hypermethylation in gene promoter regions has been implicated in tumorigenesis through the epigenetic silencing of key tumour suppressor genes (Sadikovic et al., 2008; Sharma et al.,2010). Many tumour suppressor genes silenced by DNA hypermethylation have been well characterized, these include: BRCA1, RASSF1A, CCBE1, OPCML, MGMT, HOXA9, and hMLH1 (Kawakami et al., 2011; Lonning et al., 2011; Gloss and Samimi, 2014; Dong et al., 2016). Chmelarova *et al* reported P53 promoter methylation in 51.5% of ovarian cancer (Chmelarova et al, 2013). These genes are involved in various cellular processes such as DNA repair, cell cycle, cell adhesion, apoptosis and angiogenesis which are all important in cancer development, and the hypomethylation of repeated DNA sequences leading to over expression of tumour causing genes known as oncogenes, thereby causing genetic instability by promoting chromosomal re-arrangement as shown in **Figure**

1.5 (Liu et al., 2009; Sharma et al., 2010; Earp and Cunningham, 2015). Epigenetic silencing is thought to also contribute to tumour initiation acting as the second hit in Knudson's two-hit hypothesis of tumour suppressor gene inactivation (Jones and Laird, 1999; Sharma et al., 2010). Interestingly, the degree of hypomethylation is correlated with tumour grade, stage and aggressive clinical characteristics in many cancers such as liver and colon (Daskalos et al., 2009; Sunami et al., 2011).

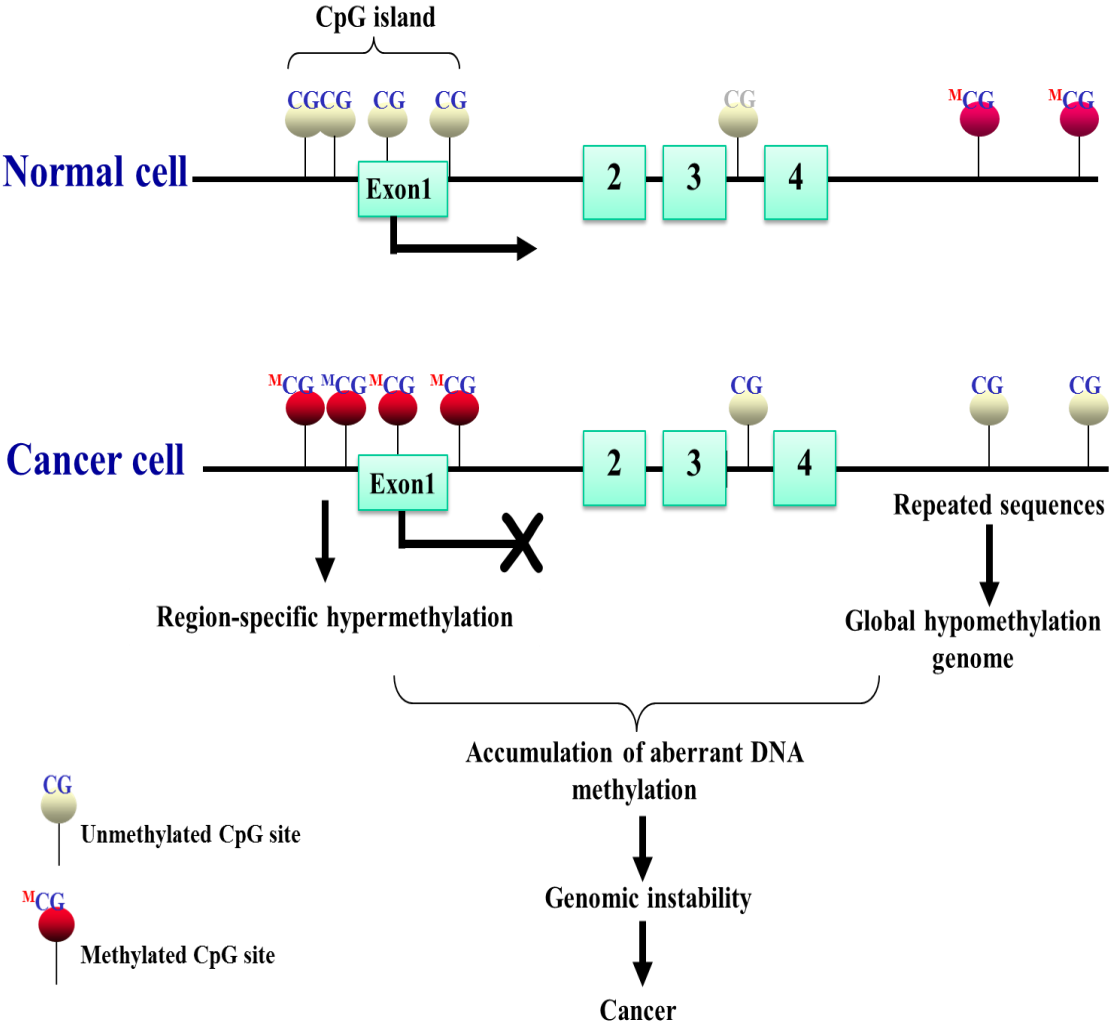


Figure 1.5 Typical DNA methylation profiles in normal mammalian and cancer cells. A global DNA hypomethylation of the genome in cancer cells at repeated sequences is observed together with DNA hypermethylation at specific gene-promoter region, such as tumour suppressor genes with silencing the corresponding gene. This pattern may lead to genomic instability and/or loss of, or reduced, transcription and following expression.

In contrast, global hypomethylation is thought to play a significant role in tumorigenesis through the activation of proto-oncogenes (Sharma et al., 2010). Several genes have been identified including H-RAS, a gene involved in cell proliferation. Consequently, this in addition to the increase in genetic instability that is cause by global hypomethylation, all may potentially contribute to the development and progression of cancer (Sharma et al., 2010).

In addition, mutations and epigenetic dysregulation of TET1 have been linked to leukaemia (Ko et al., 2010). Additionally, TET1overexpression has been found to have an oncogenic role in acute myeloid leukemia (AML) with MLL gene re-arrangement (Huang et al., 2016). Hypermethylation of promoter TET1 was found to be associated with increased DNA methylation in most of its target genes (Ko et al., 2010). Hsu *et al* and Sun *et al* found that TET1 is an essential tumour suppressor in prostate and breast cancers (Hsu et al., 2012; Sun et al., 2013). Generally, cancer cells have lower levels of 5hmC vs. normal cells in various cancers. However, it still unknown whether the loss of 5hmC is a cause or a consequence of tumorigenesis. Scourzic *et al* suggested that abnormal patterns of DNA methylation during differentiation and ageing processes might be a factor in carcinogenesis (Scourzic et al., 2015).

1.4 Oxygen, niche and hypoxia

Oxygen is one of the most important factors in the life of many organisms (Konigsberg et al., 2013). In mammals, oxygen plays a key role in regulation of many cellular functions such as cell metabolism, cell division and differentiation (Ivanovic, 2009; Konigsberg et al., 2013). Oxygen concentrations vary widely across different human tissues, but in general, are much lower than the 21% oxygen in atmosphere air. However, in a manner which displays

dependence on the distance away from the vasculature, the oxygen tension can range from between 0.1% - 12% with an average of 2% O_2 (Stamati et al., 2011; Jez et al., 2015). For example, oxygen level ranges from 0.5% to 7% in the brain (Hemphill et al., 2005). In the bone marrow ranges are between 0.5% to 4% (Chow et al., 2000) while in the eye it can vary from 1% to 5% (Yu and Cringle, 2005). The human embryo is initially developed in an environment (female reproductive tract) where the oxygen level is far lower than that of air oxygen condition (Adam et al., 2004). It is reported that oxygen tensions of 5-10% or lower enhances development of embryos from different species (Karja et al., 2004). In contrast, higher oxygen is found to be harmful to human embryos probably due to formation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide that causes DNA damage, alter gene expression and induce epigenetic modifications during embryonic development by affecting kinases and gene transcription (Yuan et al., 2003; Meintjes et al., 2009; Donkena et al., 2010). However, *in vitro* it is reported that development of embryos depends on the quality of culture environment to which embryos are exposed to (Yuan et al., 2003). Also, Forsyth *et a*l found that reduced oxygen tension in the ranges 2-5% are associated with reduced telomere shorting and enhanced proliferation in culture primary mortal fibroblasts (Forsyth et al., 2006).

However, the cell "niche" concept was first proposed by Schofield in 1978 and refers to the particular microenvironment where stem cells reside *in vivo* (Schofield et al., 1978; Papagiannouli and Lohmann, 2015). The stem cells can reside in perivascular niches which exhibit low oxygen tensions, even with cells that associate closely with blood vessels such as mesenchymal stem cells, found with bone marrow (ranges 1-7% O_2), which is lower than the oxygen level in peripheral blood (Mohyeldin et al., 2010; Sheehy et al., 2012; Jing et al., 2012). Recent reports have confirmed that the communication and interaction between stem cell niche elements and architecture including cells, the oxygen tension, blood vessels,

matrix glycoproteins and the three-dimensional space is important for stem cell self-renewal and differentiation (Mohyeldin et al., 2010). The stem cell niche maintains the cells primarily in a quiescent condition through inhibit of cell proliferation and maintenance of a low metabolic state via specific signals provision. However, when there is demand, cells respond to altered signals quickly via disruption of the homeostatic balance between symmetric and asymmetric divisions (Li and Neaves, 2006; Gattazzo et al., 2014). However, reduced oxygen can occur under many physiological conditions such as normal embryonic development and many human pathological conditions, including cancer (Semenza, 2010; Damaki et al., 2012). In addition, the discovery of the hypoxia inducible factor (HIF) as a major regulator of the cellular response to hypoxia opened new windows for researchers to investigate the critical role of this factor in regulation of many cellular processes by changing the transcriptional activity of genes and protein production (Adamaki et al., 2012). Normoxia is referred to air oxygen (21% O_2) whilst physiological normoxia/hypoxia refers to a condition where the oxygen level inside the human body is significantly lower than that in the atmosphere (between 1% and 5%,) (Loicono and Shapiro, 2010; Zhi et al., 2018).

1.4.1 Mechanism response to reduced oxygen (hypoxia)

The mechanism of oxygen level driven modulation of cellular behaviours is linked to the Hypoxia inducible factors-1A (HIF1A). HIFs consists of three isoforms: HIF1A, HIF2A and HIF3A, whereas HIF-1β, also known as ARNT (aryl hydrocarbon receptor nuclear translocator), has only one isoform, HIF-1β. Three alpha subunits (HIF1A, HIF2A, and HIF3A) all bind to a common subunit HIF-1β (Gordan and Simon, 2007). In brief, under air oxygen (normoxia) HIFα is hydroxylated on two conserved proline (P402 and P564) residues by a prolyl-hydroxylases family member (PHD1, PHD2, and PHD3). HIFs are regulated by Von Hippel Lindau protein (pVHL) which marks hydroxylated HIFs for ubiquitination and degradation by proteasome 26S as shown in **Figure 1.6**. In contrast, under

hypoxic conditions hypoxia stabilises HIF1A because the hydroxylation by PHDs is prevented, resulting in accumulation of HIF1A in its stable form, translocation into the nucleus, and binding to the HIF-1β to form the active HIF dimer (Fandrey et al., 2006; Simon and Keith, 2008). HIF1A binds to a specific DNA sequence present in target gene promoters, known as the hypoxia responsive element (HRE), and activates a group of genes that promote adaptation and survival (Ke and Costa, 2006; Luo et al., 2011).

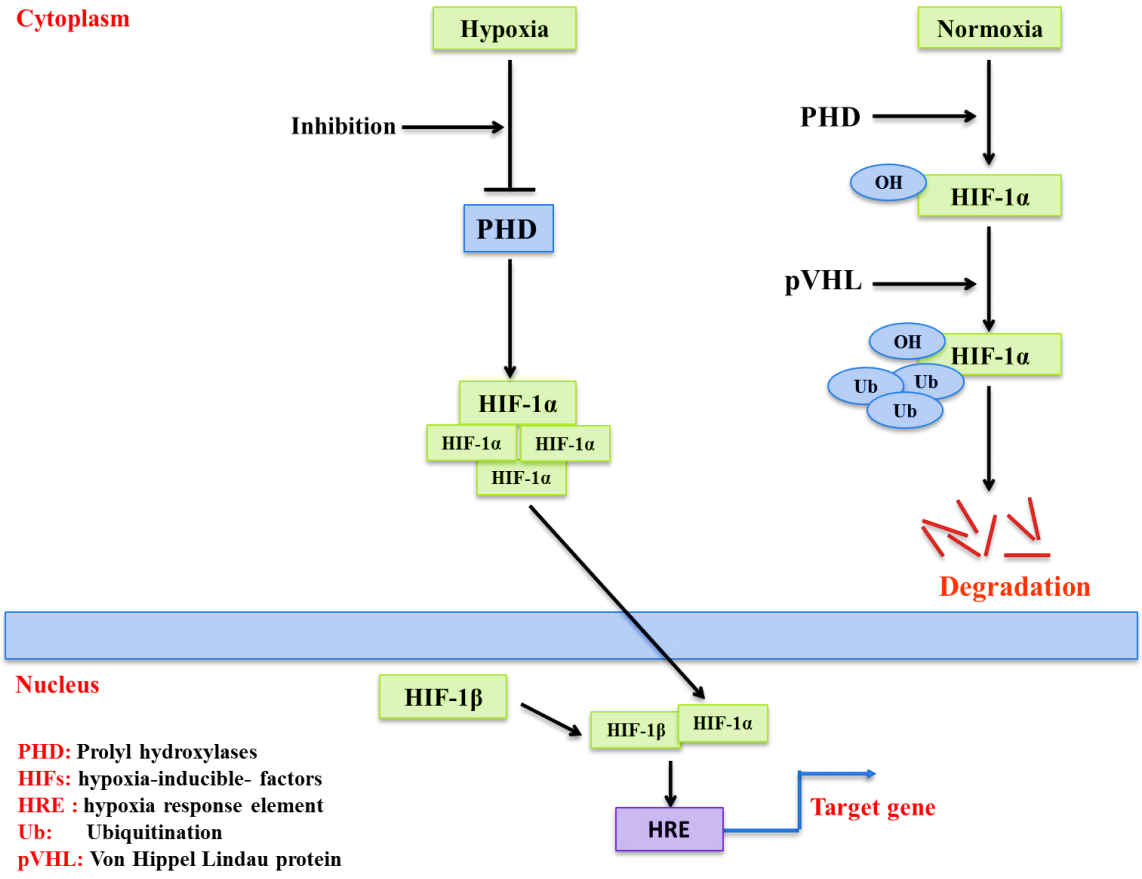


Figure 1.6 Hypoxia inducible factors up-regulation pathway. In normal oxygen tension Von-Hippel Lindau (VHL) tumour suppressor protein (pVHL) binds to and target HIF for degradation by ubiquitination. On the other hand, in hypoxic condition pVHL does not bind to HIF, consequently its levels increase within the cell, where it then subsequently binds to downstream hypoxia response elements within the nucleus resulting in the upregulation of the expression of genes that facilitate its survival within hypoxic conditions.

1.4.2 Low oxygen regulates pluripotent embryonic stem cells

The ability of stem cells to self-renew or differentiate depends on various micro-environmental cues including support cells, oxygen tension, soluble growth factors, extra-cellular matrix, and mechanical forces (Mohyeldin et al., 2010; Abdollahi et al., 2011). In the physiological setting, oxygen tension is much lower than the 21% O_2 found in atmospheric air. In general and depending on the tissue type and where the cells reside, oxygen may vary from 12% in peripheral blood to virtually anoxic in deep areas of cartilage zones (Amorin et al., 2013). However, Ezashi *et al* were the first to demonstrate that hESCs spontaneous differentiation decreased in 3%-5% O_2 vs. atmospheric oxygen (Ezashi et al., 2005). Simultaneous to these reports it was noted that 2% O_2 was more physiological and was optimal for undifferentiated hESC proliferation and reduced the spontaneous chromosomal abnormalities noted in early and late passage cultures (Forsyth et al., 2006; Ludwig et al., 2006). These observations were supported by Närvä *et al* who confirmed that low oxygen led to the maintenance of pluripotency, prevents spontaneous differentiation, and enhanced hESCs self-renewal (Närvä et al., 2013). In addition, continuous low oxygen tension culture of hESCs led to increase expression of SSEA-3 which is a surface antigen that decreases rapidly in response to differentiation (Närvä et al., 2013). Furthermore, it has been reported that hypoxia is involved in the activate regulation of molecular pathways which govern self-renewal including OCT-4 (Simon and Keith, 2008; Mohyeldin et al., 2010). In contrast, other researchers have revealed that low oxygen may be a potent stimulus for differentiation into specific cell lines (Koay and Athanasiou, 2008). More information is needed to understand the optimal oxygen concentration required to stimulate differentiation versus maintenance of stemness (Koay and Athanasiou, 2008; Abdollahi et al., 2011). Many studies now suggest that hypoxia-inducible factors, HIF-1A and HIF-2A, are master regulators of the stemness properties of stem cells and as stimuli for stem cell differentiation

(Wenger, 2002; Fong and Takeda, 2008; Mimeault and Batra, 2013). In recent study, Zhi *et al* found that increased HIFs expression under low oxygen (10% O_2) enhanced the stemness properties and significantly increased the proliferation ability of induced hepatic stem cells by quickening G1/S transition via p53-p21 signalling pathway (Zhi et al., 2018).

1.4.3 The effects of physiological normoxia on mesenchymal stem cells

The first experiments designed to test the impact of different oxygen concentrations were performed by Zwartouw and Westwood in 1958. They observed that the proliferation of cells was more rapid in hypoxia conditions when compared with the normoxic state (Zwartouw and Westwood, 1958). In mammals, mesenchymal stem cells can be found in perivascular niches that are associated closely with vascular structure. In general, the local oxygen level in different tissues where MSCs reside is very low. Therefore, it has been hypothesized that cultured MSCs in low oxygen tension leads to an improvement in MSCs survival and proliferation as well as in the maintenance of typical MSC features (Amorin et al., 2013). Many studies have shown that low oxygen culture of MSCs has a significant effect on their expansion, delayed senescence, and formation of colony forming units- fibroblasts (CFU-F) in contrast to air cultured (Hung et al., 2007; Potier et al., 2007; Berniakovich and Giorgo, 2013; Kay et al., 2015). Opposite to these findings, other studies have reported that there is no difference in the growth rate of MSCs or multipotency when maintained in both lower oxygen level and atmospheric conditions (Malladi et al., 2006; Wang et al., 2011). However, similar arguments have been raised about the potential of hypoxia to impact on tri-lineage differentiation of MSCs. For example, Huang et al found that hypoxia state increased differentiation potential to all three lineages in rat MSCs (Huang et al., 2013). Other studies indicated that low oxygen tension reduced MSCs differentiation into osteogenic and adipogenic lineages (Volkmer et al., 2010; Yang et al., 2011). These contradictory results are largely due to the difference in oxygen concentration and cell type (Abdollahi et al.,

2013). However, BM-MSC, culture in low oxygen (2% O_2) for long term led to increase the expression of embryonic markers such as OCT-4 (Grayson et al., 2007). Wang *et al* reported that a 5% O_2 but not 2% or 10% O_2 enhanced the differentiation of adipose tissue-derived stem cells (ASCs) to form SMCs with typical functional of SMCs *in vitro* (Wang et al., 2018). In addition, low oxygen conditions (2% O_2) stimulated the bioactivities of MSCs such as proliferation, wound-healing and migration capacity via the HIF1A-GRP78-Akt axis pathway (Lee et al., 2017). Furthermore, many studies suggest that low oxygen enhances plasticity, survival, proliferation, engraftment, and genetic stability of MSCs (Haque et al., 2013; Beegle et al., 2015; Huang et al, 2016). However, suitable oxygen tension, optimum incubation time, paracrine action, and the underlying mechanism need further investigation (Lee et al., 2017).

1.4.4 Tumour Hypoxia

It is well-recognized that the development and progression of many cancers are influenced by the interactions between cancer cells and their local tumour microenvironment (Hockel and Vaupel, 2001). Oxygen levels in normal tissues are maintained under strict controls to ensure normal cellular function, however, oxygen tensions within tumours can range from physiological levels of ~7% to severely hypoxic tensions of <0.1% (Heddleston et al., 2010; Thirlwell et al., 2011). Hypoxia can arise under many pathological conditions including cancer when metabolic demands for oxygen exceeds supply as vascular formation cannot keep up with the rate of cancer growth (Semenza, 2010). A study of cancer cells under hypoxia conditions indicated abnormal cell proliferation, aberrant blood vessel formation via upregulation of angiogenic factors, and metastatic mechanisms (Majmunder et al., 2010; Watson et al., 2010). In addition, hypoxia alters cancer cell metabolism and contributes to therapy resistance by inducing cell quiescence. Enhancing or reducing the positive or negative hypoxic effects on cancer cells could be due to a number of complex intracellular

pathways interacting with each other such as HIF, PI3K, MAPK, and NFκB signalling. These pathways can affect many cell processes including cell proliferation, survival, cell death, metabolism, migration, and genetic instability. In addition, the HIF pathway is needed in physiological processes and is involved in cancer biology by controlling hundreds of genes (Beatout et al., 2008; Muz et al., 2014). One such example is vascular endothelial growth factor (VEGF) which promotes angiogenesis (Hockel and Vaupel, 2001; Thirlwell et al., 2011). Interestingly, this pathway can also be activated as a result of DNA methylation within the VHL promotor region, which subsequently results in the transcriptional repression and the unopposed activation of HIF (Thirlwell et al., 2011). A recent study shown that induction of the HIF pathway by inhibition of the prolyl hydroxylase (PHD) family improved blood vessel abnormalities in hypoxic tumours and consequently improved the sensitivity to chemotherapeutic drugs by activation transcription of genes that involved in angiogenesis (Koyama et al., 2017).

Furthermore, HIF1A is also involved in down-regulation of some genes such as DNA repair (REV3), mRNA processing (hnRNP H1) and cytoskeleton maintenance (Rho kinase) (Greijer et al., 2005). Raval *et al* reported that HIF1A and HIF2A could have opposing roles on the same hypoxic inducible genes. For example, HIF1A overexpression is involved in restricted tumour growth, whilst an increase of HIF2A expression pushed tumour progression (Raval et al., 2005). Recent reports have suggested that the hypoxia microenvironment is also involved in cancer progression via activated adaptive transcriptional programs that support many biological tumour features such as survival, motility, and tumour angiogenesis (Keith and Simon, 2007). Furthermore, genomic instability induced by tumour microenvironment, including hypoxia, leads to increased point mutations, gene amplification and fragile-site induction (Reynolds et al., 1996; Coquelle et al., 1997; Bindra et al., 2005; Luoto et al., 2013).

1.5 Hypoxia induces epigenetic marks in cancer

It has been hypothesized that hypoxic microenvironments may influence alterations in DNA methylation, resulting in inappropriate silencing or activation of genes involved in the progression of cancer (Shahrzad et al., 2007). However, HIFs are involved in regulation of gene expression to hypoxia through interaction with specific DNA sequences known hypoxia response elements (HREs) within the DNA genome (Waston et al., 2010). Furthermore, DNA methylation can impact cellular adaptation to hypoxic conditions through regulation of gene expression (Robinsons et al., 2012). However, changes in DNA methylation can influence the control of HIF binding to target genes directly by methylation of a specific CpG dinucleotide within the consensus HRE (Rossler et al., 2004). On the other hand, recent evidence has suggested that hypoxia–induced gene specific DNA hypermethylation can impact upon genes that were previously active (Waston et al., 2010).

Study by Waston *et al* revealed that cells maintained in limited oxygen (1%) displayed reduced global DNA methylation (Waston et al., 2009). However, in the absence of HIF1A the level of DNA methylation increased and was associated with significant increases in DNA methyltransferase (DNMT3B) expression (Waston et al., 2009). Further to this hypoxia-inducible transcription factor HIF1A is involved in regulation of the DNA methyltransferase enzymes DNMT1 and DNMT3B enzymes (Watson et al., 2014). In addition, studies have shown a decrease in 5mC levels following exposure of cancer cell lines to severe hypoxic conditions (Shahrzed et al., 2007). Similarly, a decrease in 5mC levels was observed in *in vivo* xenograft cancer cells following exposure to severe hypoxic conditions (Shahrzed et al., 2007). In addition, it has reported by Mariani *et al* that hypoxia increases global 5hmC levels, with accumulation of 5hmC density at canonical hypoxia response genes and that these are associated with TET1 upregulation in hypoxia condition (Mariani et al., 2014). In contrast, Thienpont *et al* reported that low oxygen causes DNA

hypermethylation by decreasing TET activity (Thienpont et al., 2016). Generally, the interaction of epigenetics and hypoxia occurs via four mechanisms (Watson et al., 2010):

- Epigenetics controlling HIFs stabilization.
- Controlling HIFs via regulation of transcription of hypoxia response elements (HRE).
- Histone demethylase enzyme as HIFs Targets.
- Significant global changes in histone modification and DNA methylation

For example, as shown in **Figure 1.7**, silenced of PHD3 and VHL by promoter hypermethylation is possibly an important epigenetic mechanism that may involve in controlling HIFs stabilization (Hatzimichael et al., 2009).

However, better understanding of the role of hypoxic microenvironment in the field of cancer research, especially in DNA methylation area will open new windows for the discovery new therapeutics targeting the cancer cells (Muz et al., 2015).

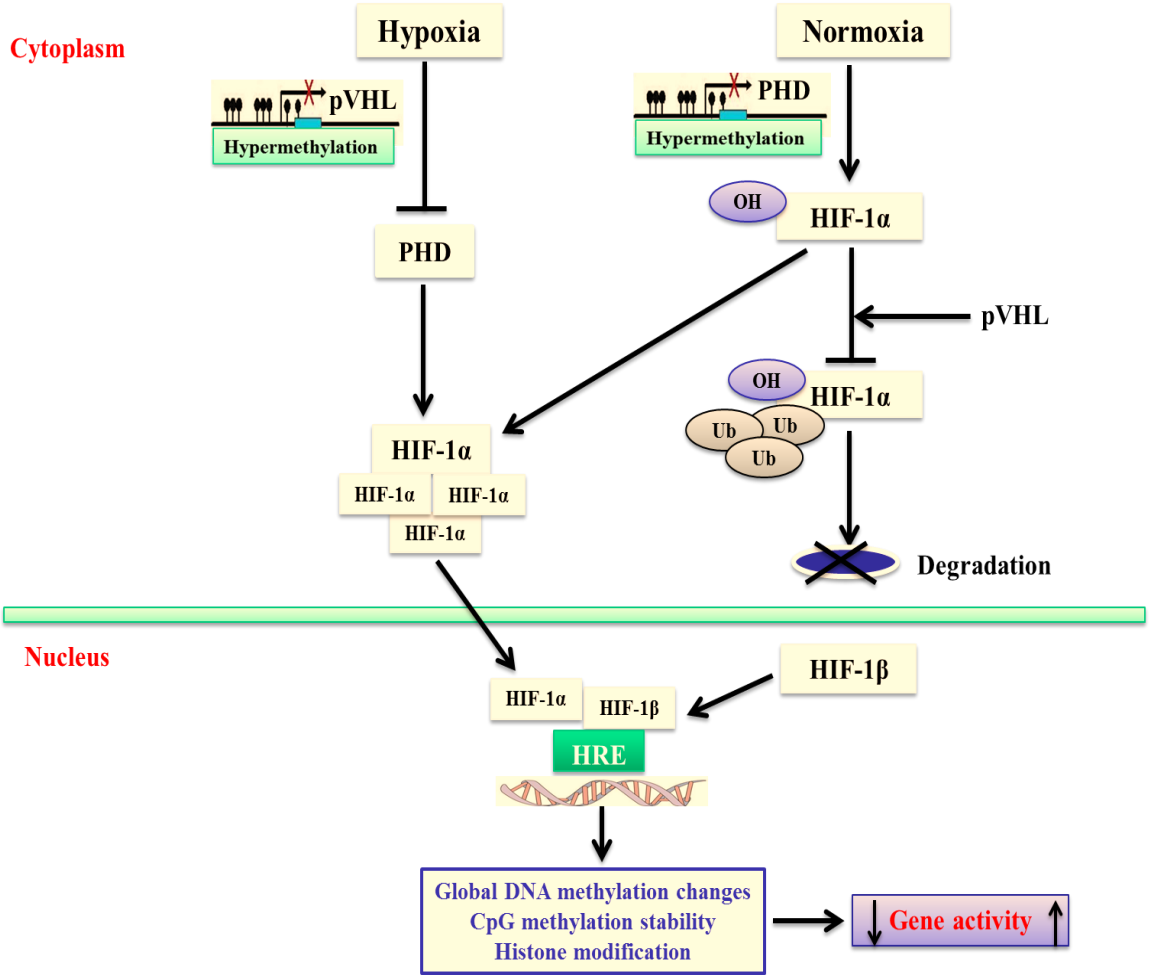


Figure 1.7 key interactions between important epigenetics mechanisms and hypoxia. This figure summarises the role of epigenetics in controlling HIFs stabilization. Silenced of PHDs and VHL by promoter hypermethylation led to induce upregulation of HIFs, drives the activation of several target genes. Hypoxic conditions modulate genes expression profile via several mechanisms, included global DNA methylation alterations, local CpG changes on HIFs target genes and histone modification.

1.6 O_2 distribution in the normoxic and hypoxic incubators

It has become increasingly clear that cultivating cells *in vitro* not only temperature, humidity, and CO_2 but also O_2 needs to be controlled. However, the actual normoxic O_2 concentrations are almost never correctly indicated but rather given as 21% O_2, which the equal to the O_2 concentration of room air rather than incubator air. Moreover, the O_2 concentration in the gas phase, it will never be what the (adherent) cells in a tissue culture dish experience, since

they are attached to the bottom of the dish. Importantly, O_2 solubility in the aqueous phase is rather low (Wanger et al., 2015). The mechanism by which gases reach the bottom of the tissue culture dish or flask is by diffusion, which is almost always the limiting factor for cellular oxygenation. After moving the dish out of a normoxic incubator and exposing it to room air conditions, the environmental O_2 supply acutely increases and a shallow O_2 gradient forms due to the poor O_2 diffusion in unstirred medium, even in the absence of cells (Pettersen et al., 2005; Polak et al., 2015). However, the onset of hypoxic exposure is usually defined as the moment when the doors of the hypoxic incubator are closed, and it will take several minutes to several hours until the medium O_2 concentration asymptotically approximates the desired value, even if the incubator would change the gas phase composition rapidly. Moreover, in the absence of cells reveals a duration of 38 minutes, 45 minutes, and 60 minutes to fall below a O_2 value of 1.2-fold of the input value if a cell culture dish is acutely switched from 20% O_2 to 2%, 1%, or 0.2% O_2 concentration, respectively. However, without stirring, this will result in little change in the overall O_2 content since only the surface region releases O_2. Gas exchange with room air occurs almost instantaneously, and it will take up to 1 hour until hypoxic conditions in the incubator's gas phase are re-established. It is always better to culture, harvest, and lyse the cells within hypoxic workstations (Jaakkola et al., 2001; Wanger et al., 2015).

However, typical cell culture methods involve isolating cells from a physiological state and then analysing them in "bench-top conditions". This creates stress for the cell and introduces unknown outcomes in cell expression and morphology. So, we used the physiological workstations to mimic '*in-vivo*' conditions providing a continuous cell culture environment which eliminates cellular stress linked to variations in temperature, pH and oxidation. In addition, to address the differences between continuous vs semi-continuous low oxygen exposure, a pathological hypoxia model was also created by culturing cells in a hypoxia

incubator with intermittent air flushing due to open/shut phase of the incubator doors with all culture processing being performed under an atmospheric environment.

1.6 Aims and Objectives

The overall aims of this project are to:

1. Investigate the effects of reduced oxygen on characteristics of cells including proliferation, expression of core pluripotency factors and HIFs.
2. Investigate the effects of physiological normoxia on global DNA methylation (5mC and 5hmC) in cells.
3. Assess the relationship between changes in global DNA methylation (5mC and 5hmC) patterns in response to oxygen modulation and the transcriptional expression of DNMTs/TETs.
4. Investigate the effects of hypoxia on the local DNA methylation patterns at selected CpG sites within the promoter regions of DNMTs/TETs genes.

To test this hypothesis, this project will adopt an epigenetic approach to explore and identify epigenetics marks from hypoxic cancer cells, hPSCs and BM-hMSCs.

Chapter 2: Materials and Methods

2.1 Materials

Table 2.1 List of materials, catalogue numbers and suppliers.

Name	Catalogue No.	Supplier
3-isobutyl-1-methylxanthine (IBMX)	I7018	Sigma-Aldrich
(3-(4,5-dimethylthiazol-2yl)-,5diphenyltetrazolium bromide	M2128	Sigma-Aldrich
Agarose	BP1356-500	Fisher Scientific
Alamar blue	DAL1025	Fisher Scientific
Alcian blue	A3157	Sigma-Aldrich
Alizarin red S	A5533	Sigma-Aldrich
Annealing buffer	979009	Qiagen
Ammonium acetate	A2706	Sigma-Aldrich
Ascorbic acid phosphate	A8960	Sigma-Aldrich
Basic Fibroblast growth factor (bFGF)	100-18B	Peprotech
BCA Protein Assay Kit	23225	Fisher Scientific
Bovine serum albumin (BSA)	BP9703-100	Fisher Scientific
4′,6-Diamidino-2-phenylindole (DAPI)	D9542	Sigma-Aldrich
DNeasy Blood & Tissue Kit	69504	Qiagen
Dexamethasone	D2915	Sigma-Aldrich
Direct Load Wide Range DNA Marker	D7058	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	D2650	Sigma-Aldrich
Donkey anti-goat IgG	NL003	R&D system
Donkey serum	D9663-10ML	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM) – 4.5 g/L glucose	BE12-709F	Lonza
EDTA (0.5 M), pH 8.0	AM9260G	Fisher Scientific
Essential 8™ Medium	A1517001	Life Technologies
Ethanol (absolute)	E0650/17	Fisher Scientific
Ethidium bromide	E1510	Sigma-Aldrich
Ethylene diamine tetra-acetic acid (EDTA)	BP2482-1	Fisher Scientific

EZ DNA Methylation-Gold™ Kit	D5005	Zymo research
FBS (foetal bovine serum)	DE14-801F	Lonza
Fibronectin	F0895	Sigma-Aldrich
Flow cytometry staining buffer	FC001	R&D system
Gel Loading Buffer (0.05% bromophenol blue, 40% sucrose, 0.1 M EDTA, 0.5% SDS)	G2526	Sigma-Aldrich
Glycine	G8898	Sigma-Aldrich
Go Taq G2 Flexi DNA polymerase	M7801	Promega
Goat anti-mouse IgG	NL557	R&D system
Hepes free acid	B30487-1KG	VWR
Human Pluripotent Stem Cell Marker Antibody Panel kit	SC008	R&D system
Indomethacin	I7378	Sigma-Aldrich
Insulin	I9278	Sigma-Aldrich
Isopropanol	P/7500/17	Fisher Scientific
Knockout DMEM	10389172	Fisher Scientific
Leupeptin	L8511	Sigma-Aldrich
Live human stem cell imaging Kit	SC023	R&D system
L-Glutamine	BE17-605E	Lonza
L-Proline	P5607	Sigma-Aldrich
Methanol	M/3900/17	Fisher Scientific
MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit	P-1036	Epigentek
MethylFlash Methylated DNA Quantification Kit	P-1034	Epigentek
Non-essential amino acids NEAA	BE13-114E	Lonza
Oil Red O	O0625	Sigma-Aldrich
Nonidet P-40	56741-50ML-F	Sigma-Aldrich
One Step RT-PCR Kit	210210	Qiagen
Paraformaldehyde	P/0840/53	Fisher Scientific
Pepstatin A	P5318	Sigma-Aldrich
Penicillin, streptomycin, amphotericin B	BE17-745E	Lonza
Phenylmethylsulfonyl fluoride (PMSF)	P7626	Sigma-Aldrich

Phosphate buffered saline (Mg2+ and Ca2+ free)	BE17-516F	Lonza
Phycoerythrin conjugated antibodies CD14 CD19 CD34 CD45 HLA-DR CD73 CD90 CD105 IgG1 IgG2a	130-098-167 130-098-168 130-098-140 130-098-141 130-098-177 130-097-943 130-097-932 130-098-906 130-098-845 130-098-849	Miltenyi biotec
PyroMark Gold Q24 reagents	970802	Qiagen
QIAshredder mini spin columns	79656	Qiagen
Quantifast SYBR green RT-PCR kit	204154	Qiagen
RNase A	19101	Qiagen
RNeasy mini kit	74104	Qiagen
Sodium Chloride	S7653	Sigma-Aldrich
Sodium deoxycholate	30970-25 g	Sigma-Aldrich
Sodium dodecyl sulphate SDD	L4390	Sigma-Aldrich
Sodium pyruvate	S8636	Sigma-Aldrich
Streptavidin sepharase	17-5113-01	GE Healthcare
TE buffer	P7589	Invitrogen
Transforming growth factor B3 (TGF-B3)	100-36E	Peprotech
Tris-Hepes NH 4-20%	NH31-420	Nusep Ltd
Triton X-100	9002-93-1	Sigma-Aldrich
Trypan blue	T8154	Sigma-Aldrich
Trypsin	15090	Life Technologies
Tween 20	66368	Analar
Uplight substrate	S83728	Interchim
Vitronectin (VTN-N) recombinant human protein, truncated	A14701SA	Life Technologies
β-glycerophosphate	G9422	Sigma-Aldrich
β-mercaptoethanol	10368072	Fisher Scientific

2.2 General cell culture techniques

2.2.1 Cell culture conditions

All cells were maintained in the following three oxygen conditions:

- 21% O_2-Standard cell culture (21%ST)
- 2% O_2-Pre-gassed media in a 2% O_2 incubator (2%PG)
- 2% O_2-Pre-gassed media in a 2% O_2 Workstation (2%WKS)

21%ST cells were cultured in humidified air and 5% CO_2 incubator and handled in a standard biological safety laminar flow cabinet. For reduced oxygen cells were grown in either a nitrogen-regulated tri-gas controlled incubator set at 2% O_2, 5% CO_2 (2%PG) or in a tri-gas incubator set at 2% O_2 in the presence of 5% CO_2 but handled in a hermetically sealed hypoxic workstation (2%WKS) (SCI-TIVE, Baker Ruskinn Ltd, UK). The cells were then observed regularly under inverted microscope (Nikon TS100, Japan) to ensure they were healthy and to check for confluency. Mycoplasma testing was performed across randomly selected cell lines by Guy Hilton technician team to ensure that there was no contamination. All cell lines were incubated at 37^{o}C. Surprisingly, many epigenetics studies have focusing on short term hypoxic effects (24h and 3 days) and there are yet no reports deal with long term hypoxic effects. However, all cell types that we used in this study were culturing at least for five passages under both hypoxic conditions (2%PG and 2%WKS) prior to experiments.

2.2.2 Hypoxic media

To maintain the oxygen level of the cells during the physiological oxygen culturing, cell culture media was deoxygenated to a 2% O_2 level using a HypoxyCool unit (Baker Ruskinn

Ltd, UK). The unit was pre-set to deoxygenate the supplemented medium to predefined oxygen concentrations (2% O_2). To maintain sterility, avoid the risk of contamination during gas exchange, and allow for the fast reduction of the dissolved oxygen levels within the media, the media bottles caps (ThermoFisher) were replaced with vented closure caps within a sterile hood before the bottles were placed within the HypoxyCool system (**Figure 2.1)**. To monitor the big pH changes during the culturing our cell with different oxygen levels, we used the cell culture media contains phenol red as an indicator for pH changes that will turn yellow at low pH and purple at high pH level. However, the effect of reduce oxygen (3% O2) on the pH levels was evaluate in new study by using the pH-sensitive dye BCECF, the results showed that hypoxia has little effect (0.05) on steady-state pH level. Also, Jørgensen *et al* study showed no significant change in pH levels during chemical anoxia in mouse neocortical neurons in primary culture (Jørgensen et al., 1999).

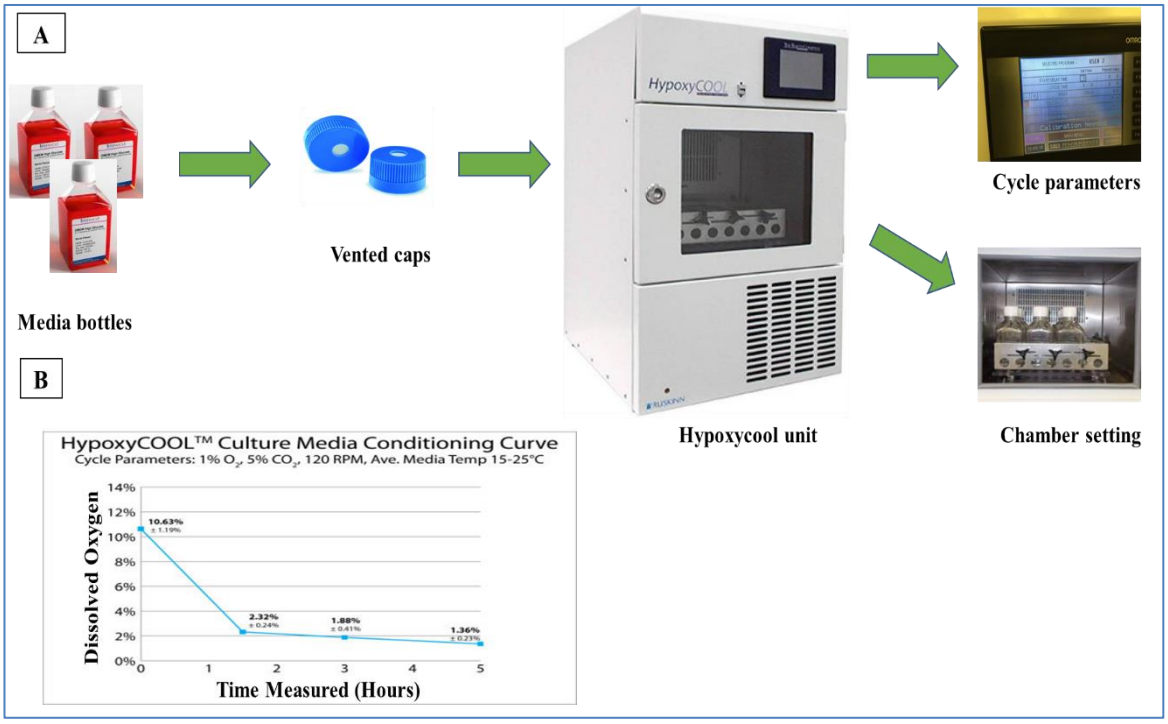


Figure 2.1 Media deoxygenation. A) The media bottles caps were aseptically replaced with vented closure caps before being placed into the Hypoxycool unit. The cycle parameters used to establish dissolved oxygen levels within the media were as defined by manufacturors

instruction. B) Oxygen deoxygenation within the media over time. Source: The Baker Company.

2.2.3 Enzymatic sub-culture and expansion

When cells had reached 80-90% confluence the media was aspirated, and cells washed with calcium and magnesium free PBS. After washing cells were exposed to either 1% Trypsin/PBS (Embryonic stem cells) or to 0.5mM of ethylene diamine tetra acetic acid (EDTA) (BM-hMSCs and Cancer cells) in PBS solution and culture vessels were incubated for 3-5 minutes at 37°C until cells began to detach. Following detachment 1ml of pre-warmed media was added, and the cells removed by gently pipetting media up and down onto the plate surface to create a cell suspension. Cell suspensions were then centrifuged at 1000 rpm for 3 minutes to pellet the cells, supernatant removed, and the pellet re-suspended in fresh supplemented media in preparation for seeding at the correct dilution or cells/cm^2 as experimentally required. This procedure was performed for all cells cultured within the three different oxygen conditions; 21%ST, 2%PG and 2%WKS.

2.2.4 Cryopreservation of cells

Following detachment as described in **Section 2.2.3** cells were re-suspended in 1ml cryopreservation solution comprising 10% dimethyl sulphoxide (DMSO) in fresh media and immediately transferred into a cryovial. The cryovial was then placed into a Mr Frosty container and then into a -80^{o}C freezer overnight before transfer into a suitable liquid nitrogen vessel for long term storage.

2.3 Culturing and cell characterization of human pluripotent stem cells

2.3.1 Cell lines, sub-culturing and expansion

Two human ES cells (SHEF1, SHEF2) were obtained from the laboratory stock (Guy Hilton Research Centre) and used under approval from the UK Stem Cell Bank (UKSCB) (Aflatoonian et al., 2010). Human induced pluripotent stem cell line (iPS) (ZK2012L) was kindly provided by Professor. Susan Kimber and Dr. Zoher Kapacee, Faculty of Biology Medicine and Health, University of Manchester. The ZK2012L cell line was derived from human dermal fibroblasts (formerly in the Kimber lab). As per methods of Takahashi and Yamanaka, and Takahashi *et al* protocol (Takahashi and Yamanaka, 2006, Takahashi et al., 2007), the human dermal fibroblasts were transduced with retroviruses carrying the genes encoding for Oct 4, Sox 2, Klf 4 and c-Myc for 24 h then re-plated on a plate seeded with a feeder layer of iMEFs. Twenty-five to thirty days after transduction, the colonies formed were manually dissected, re-plated (Cheng et al., 2014). Cells were routinely cultured in the defined, Xeno-free, E8 system (Life Technologies). Media was prepared by first slowly thawing 10ml Essential 8 Supplement (50x) at 2-8^{o}C overnight which was then mixed with 490ml Essential 8 basal media to prepare 500ml of complete Essential 8 (E8) basal medium. Cells were thawed rapidly in a water bath (37^{o}C) with gentle agitation following removal from liquid nitrogen. Once thawed the contents of the cryovial were added to 4ml of complete Essential 8 (E8) basal medium, centrifuged at 1000 rpm for 3 minutes, and the supernatant removed. The cell pellet was then re-suspended in 2ml of medium and seeded into one well of a 6 well plate coated with vitronectin (VTN-N) Recombinant Human Protein, Truncated, Prototype; Life Technologies). Coating was performed by adding 60µl (rhVTN-N) to 6ml of Dulbecco's phosphate-buffer saline (DPBS) without calcium and magnesium (Gibco) to prepare 5mg/ml working vitronectin solution and then 1ml was added to each well of a 6-well tissue culture plate followed by incubation at room temperature 1

hour before use. After incubation the vitronectin solution was discarded and 2ml of hPSCs culture media added. hPSCs were cultured within the three different oxygen conditions; 21%ST, 2%PG and 2%WKS. hPSCs were fed with new Essential 8 basal medium every day and cells were sub-cultured enzymatically at confluence using 0.5mM EDTA as described in **Section 2.2.3**.

2.3.2 Spontaneous differentiation

hPSC were grown in 6-well vitronectin-coated culture plates in three conditions as described above for 48 hours in E8 medium before switching into spontaneous differentiation medium (Knockout DMEM, 10% FBS, 1% NEAA, 1% L-glutamine and β-mercaptoethanol) as described in previously work (Kumar et al., 2015). The medium was changed every three days. Differentiated cells were characterised at three different time points; days 5, 10 and 20.

2.3.3 Characterisation of human pluripotent stem cells

2.3.3.1 Immunocytochemistry

Undifferentiated hPSC were seeded at a density of 3.5 x 10^4 cells/cm^2 to each well of a 6 well plate and maintained in Essential 8 basal Medium over 96 hours prior to immunocytochemistry. Cells were characterised using the unfixed live human stem cell imaging kit (R&D systems, UK). The medium was aspirated, washed twice with PBS before adding 1ml fresh Essential 8 basal media containing 1x concentration (1:50) of antibody (SSEA-4, TRA-1-60, and SSEA-1) followed by 30 minutes incubation at 37^{o}C. Then, the antibody solution was removed, hPSCs washed with fresh media, and re-fed with fresh Essential 8 Medium. Labelled cells were immediately imaged using fluorescent microscopy (Nikon Eclipse Ti-S, Japan) with a Nikon digital slight DS-Qi1Mc camera.

2.3.3.2 Flow Cytometric analysis of cells (FACS)

Approximately 5 x 10^5 cells were labelled with hPSC markers of pluripotency (SSEA-4, TRA-1-60 and SSEA-1) antibodies as described above. Media was aspirated, cells washed twice with PBS, and cells harvested with Trypsin-versene (EDTA) and centrifuged at 1000 rpm for 3 minutes. The supernatant was removed, and the cell pellet washed in flow cytometry buffer twice with a centrifugation at 1000 rpm for 3 minutes on each occasion. The cell pellet was then re-suspended in FACS buffer ((PBS with 0.5% (w/v) BSA (Fisher Scientific) and 2mM EDTA (Fisher Scientific)) and run into the flow cytometer (Beckman Coulter Cytomics FC 500). At least 1-3 x 10^4 events were acquired, and the data analysis was carried out using flowing software version 2. The percentage of positive events were determined by using gates to exclude 99% of the appropriate control events. Data analysis was performed with Flowing Software and the expression of SSEA-4 determined in the FL1 channel, while TRA-1-60 and SSEA-1 were in the FL2 channel.

2.3.4 Evaluation of pluripotent markers expression in human pluripotent stem cells and their progeny

Spontaneous differentiation of hPSCs was assessed using Human Pluripotent Stem Cell Marker Antibody Panel kit (R&D systems). Cells were seeded in 24 well-plates using E8 medium coated with vitronectin before being transferred into spontaneous differentiation media as described in **Section 2.3.2**.

Spontaneous differentiation was examined in undifferentiated cells (Day 0) and from spontaneous differentiation cells at three different time points; days 5, 10 and 20 under all conditions (21%ST, 2%PG and 2%WKS). Prior to fixation the cells were washed twice with PBS and fixed with 4% paraformaldehyde solution (in PBS). After 40 minutes incubation at room temperature, 0.1% Triton™ X-100 was added for 5 minutes to permeabilise

membranes. Fixed cells were then washed twice before incubation with blocking buffer (3% BSA, 10% normal donkey serum in PBS) for 1 hour at room temperature to block non-specific antibody binding. After washing, the cells were incubated with primary antibodies for either mouse anti-human SSEA-4 monoclonal, goat anti-human Nanog, monoclonal mouse anti-human alkaline phosphatase (ALP), and goat anti-human OCT-4 at 1µg/100µL and incubated at 4°C overnight. Followed by incubation for 2 hours at room temperature with 5µg/ml of secondary antibody specific to either OCT-4 and NANOG (donkey anti-goat IgG (R&D Biosystems)) or SSEA-4 and ALP (goat anti-mouse IgG (R&D systems). Non-specific staining of the nuclei was visualised with 4, 6-Diamidino-2-phenylindole (DAPI) at a dilution of 1:500 in PBS for 5-10 minutes. Cells were immediately imaged using a Nikon fluorescence microscope (Nikon TZ1; Leica, Germany).

2.4 Isolation and culture of bone marrow mesenchymal stem cells

Three human mesenchymal stem cell (hMSCs) lines were isolated and expanded from commercially sourced (Lonza, USA) whole human bone marrow aspirate (BMA) using the plastic adherence method described by previously published techniques (D'Ippolito et al., 2003, Kay et al., 2015). T-75 tissue culture flasks were coated with 10ng/ml of fibronectin (Sigma) diluted in PBS and incubated at room temperature for a minimum of 1 hour. After that, the fibronectin solution was aspirated and 15ml of DMEM media supplemented with 5% FBS, 1% L-glutamine, 1% NEAA and 1% PSA (Penicillin-Streptomycin-Amphotericin B) (100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B) was added to each T-75 tissue culture flasks. The whole bone marrow was cultured at a density of 1 x 10^5 mononuclear cells (MNCs)/cm^2 in 75 cm^2 tissue culture flasks and incubated for one week at 37°C in three different oxygen conditions as a described in **Section 2.2.1**. Then, a 50% media change was performed and antibiotic-free fresh DMEM culture medium was added. One week later, the whole media was removed, and 20ml of complete fresh media added.

Complete fresh media changes were carried out twice weekly. Once the cells reached confluence the media was removed, and the cells washed twice with PBS and cells detached using 3ml of trypsin/EDTA in PBS solution and the cells passaged as described in **Section 2.2.3.**

2.4.1 Characterisation of mesenchymal stem cells

2.4.1.1 Tri-lineage differentiation

In order to determine the differentiation potential of hMSCs, the cells were assessed at P2 using multipotency differentiation media. MSCs were seeded at 2 x 10^4 cells/cm^2 in 24 well plates in in complete culture media overnight to allow attachment and subsequently allowed to reach 80-90% confluency prior to being exposed to either osteogenic, adipogenic or chondrogenic differentiation media alongside controls. Media was changed twice weekly for 20 days.

2.4.1.1.1 Adipogenic differentiation

Cells were cultured in DMEM media supplemented with 10% FBS, 1% L-glutamine, 1% NEAA and adipogenesis differentiation induced with 0.5μM dexamethasone, 10μg/ml insulin (Sigma-Aldrich), 100mM indomethacin (Sigma-Aldrich) and 0.5mM 3-isobutyl-1-methylxanthine (IBMX).

2.4.1.1.2 Osteogenic differentiation

Cells were cultured in DMEM media supplemented with 10% FBS, 1% L-glutamine and 1% NEAA prior to transfer into osteogenic differentiation media consisting of 0.1mM dexamethasone (Sigma-Aldrich), 50μM ascorbic acid phosphate and 10mM β-glycerophosphate.

2.4.1.1.3 Chondrogenic differentiation

Osteogenesis differentiation was induced using 1% ITS (Sigma, UK), 50mM ascorbic acid (Sigma, UK), 40µg/ml L-proline (Sigma-Aldrich), 0.1mM dexamethasone, 10ng/ml TGF-β3 (Sigma-Aldrich) and 1% sodium pyruvate (Sigma-Aldrich).

2.4.1.2 Histological staining

hMSC were cultured in differentiation media for 20 days prior to being washed twice with PBS. For adipogenic differentiation the cells were fixed using 4% Paraformaldehyde (Sigma) in PBS for 20 minutes before being washed with PBS. Chondrogenic and osteogenic differentiated cells were fixed in 95% methanol (Sigma-Aldrich) for 10 minutes.

2.4.1.2.1 Oil Red O for Adipogenesis

Oil Red O (Sigma-Aldrich) was prepared as a 0.5% solution in 99% isopropanol alcohol (Fisher Scientific) and fresh working solution prepared by mixing 3 parts of 0.5% Oil Red O stock solution and 2 parts of dH_2O for 15 minutes and filtering with a 0.22µm syringe filter prior to use. Paraformaldehyde-fixed cells were stained in fresh working solution for 10-15 minutes at room temperature. After that the stain was separated and cells washed twice with dH_2O before imaging immediately using Olympus CKX41 microscope (Japan).

2.4.1.2.2 Alizarin Red S for Osteogenesis

Osteogenic differentiation of hMSC was performed using 2% (w/v) Alizarin Red S (Sigma-Aldrich) to detect calcium deposits. Alizarin Red S (2g) was dissolved in 100ml distilled water, mixed and pH adjusted to 4.1 - 4.3 with 10% ammonium hydroxide and filtered before use. Cells were covered with staining solution and incubated at room temperature for 5 to 15 minutes followed by washing gently under running tap water. Stained cells were observed microscopically (Olympic CKX41) before image capture using Image-Pro Insight software.

2.4.1.2.3 Alcian blue for Chondrogenesis

In order to detect glycosaminoglycan production 1g Alcian blue was dissolved in 100ml of 3% acetic acid, and pH adjusted to 2.5 with acetic acid and filtered before use. Alcian blue staining solution was added to cover fixed monolayers cells for and left for 15 minutes before the stain was removed and the cells washed gently under running tap water prior to stained cells being imaged.

2.4.1.3 Flow cytometric analysis of cells

To carry out FACS hMSC cells were first seeded in a T25 flask and maintained until 80-90% confluence. After washing twice with 5ml PBS the cells were incubated with 2.5ml trypsin/EDTA for 3-5 minutes. Cells were checked microscopically before centrifugation for 5 minutes at 900 rpm. The supernatant was aspirated, and cells re-suspended with 5ml of flow cytometry buffer and centrifuged again before the cell pellet was re-suspended in 1ml FACS buffer. Cell count was carried out and 1 x 10^5 cells aliquoted into individual 1.5ml microcentrifuge tubes, centrifuged and the supernatant removed. A panel of antibodies were diluted in flow cytometry buffer (CD14, CD19, CD34, CD45, CD73, CD90 and CD105, HLA-DR, IgG1 and IgG2a isotype controls) and added to specific cell pellets and incubation at 4°C for 15 minutes in the dark. Cells were then washed with 1ml of FACS buffer and centrifuged at 900 rpm for 5 minutes. Supernatants were discarded, and the cell pellet re-suspended in 300µl of FACS buffer and a minimum of 50000 events were recorded via analysis on the Beckman Coulter Cytomics FC 500 flow cytometer. Associated isotype controls (IgG1: CD19, CD73, CD90 and CD105, IgG2a: CD14, CD34, CD45, HLA-DR) were applied to establish positive and negative events with all experimental conditions measured. The data analysis was performed using flowing software **(Version 2.5.1)**.

2.5 Cancer cell lines

All cancer cell lines that used in this study were obtained from the laboratory stock of Guy Hilton Research Centre, Keele University. The cells were incubated at 37^{o}C at three different oxygen tensions as described in **Section 2.2.1** and detached and passaged as described in **Section 2.2.3**. The cells were then monitored daily to ensure that there was no contamination and to check for confluency.

2.5.1 MG-63 human osteosarcoma cell line

The adherent osteosarcoma cell line (MG-63) was originally derived from a fourteen -year old male patient with osteosarcoma (Billiau, 1975). The cells were maintained in T-75 flasks in DMEM media supplemented with 10% FBS, 1% NEAA and 1% L-Glutamine. These cells were incubated and passaged with 2.5% Trypsin at split ratios of 1:4 to 1:8.

2.5.2 SH-SY5Y neuroblastoma cell line

The adherent SH-SY5Y neuroblastoma cell line was derived from SK-N-SH which was originally isolated from a 4-year-old female with neuroblastoma bone marrow biopsy (Biedler et al., 1978). The cells were maintained in T-75 flasks in DMEM supplemented with 10% FBS, 1% NEAA and 1% L-Glutamine. The cells were passaged with 2.5% Trypsin and replated at a split ratio of 1:4 to 1:8.

2.5.3 COV362 human ovarian epithelial-endometroid carcinoma

The adherent COV362 cells are a cell line derived from the pleural effusion of a human ovarian epithelial-endometroid carcinoma (Berg-Bakker et al., 1993 and Domcke et al., 2013). Cells were cultured on uncoated T-75 flasks in DMEM media supplemented with 10% FBS, 1% NEAA and 1% L-Glutamine. The cells were detached and passaged with 2.5% Trypsin at split ratios of 1:4 to 1:8.

2.5.4 A549 human lung carcinoma

The adherent human lung carcinoma cell line was originally isolated from a 58-year-old Caucasian male and first established in 1972 (Giard et al.,1973). Cells were cultured on uncoated T-75 flasks in DMEM media supplemented with 10% FBS, and 1% L-Glutamine. The cells were detached and passaged with 2.5% Trypsin at a split ratio of 1:3 to 1:6.

2.5.5 Jurkat cells

The non-adherent cell line was originally derived from the peripheral blood of a 14-year-old patient with relapsing Acute lymphocytic leukaemia (ALL) (Schneider et al.,1977; Mezencev et al., 2011). Jurkat cells were maintained in DMEM media supplemented with 10% FBS, 1% NEAA and 1% L-Glutamine. The cell suspension from the T-75 flasks was transferred into a 50ml conical tube and was centrifuged at 1000 rpm for 3 minutes to pellet the cells. The pellet was re-suspended in 15ml of fresh supplemented DMEM. 5ml of cell suspension was then pipetted into three T-75 flasks, containing fresh supplemented media at a ratio of 1:3.

2.5.6 IMR-90 human fetal lung fibroblast

The non-cancer adherent IMR-90 cells (ATCC® CCL-186™) were isolated from lung tissue of a 16-week-old female Caucasian foetus. Cells were cultured on uncoated T-75 flasks in DMEM media supplemented with 10% FBS, 1% NEAA and 1% L-Glutamine. The cells were detached and passaged with 2.5% Trypsin at a split ratio of 1:3.

2.6 Cell proliferation analysis

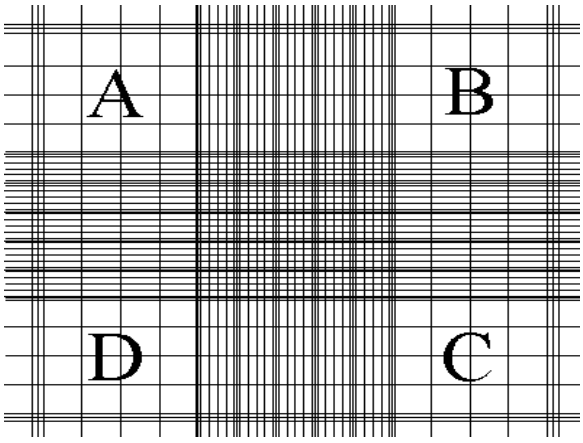
To determine the effect of long-term physiological oxygen on the growth and viability of cells, three assays of cell proliferation were utilised; quantification of cells by cell counting,

the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and Alamar Blue cell proliferation assay.

2.6.1 Cell counting

Cells were trypsined with Trypsin/EDTA (Lonza) diluted in DPBS solution to obtain a single cell suspension. Following detachment, the cells were pelleted at 200 g centrifuged, the supernatant aspirated, and the cells re-suspended in 1ml medium. Next, cells suspension was diluted 1:1 in 0.4 Trypan blue (Sigma-Aldrich) and 5µl was loaded into the haemocytometer. All viable (unstained) cells within all 4 outer squares were counted under a microscope **(Figure 2.2A)**. Total cell number was then determined by using the calculation shown in **Figure 2.2B**. Cells were counted in three independent experiments over 6 days under each condition at the same time for each day.

A



B

Cells/ml = Average of viable cells counted x 10^4

Total cells = Cell/ml x n x dilution factor

Figure 2.2 Cell count. A. Schematic of the Haemocytometer used – all viable cells within the four-outer large square were counted B. Calculation to determine the total number of cells in suspension - Average of cell count multiply by 10^4 = conversion factor to determine number of cells per ml. n= the original volume the cell suspension and the Dilution factor = dilution factor with Trypan Blue (1:1 dilution).

2.6.2 MTT assay

MTT (3-(4,5-dimethylthiazol-2yl)-2,5diphenyltetrazolium bromide) colorimetric assay was performed to evaluate cell metabolic activity. This assay quantifies the cells metabolic viability based on mitochondrial function by the action of the NAD(P)H dependent oxidoreductase enzyme conversion of the yellow MTT tetrazolium salt into purple formazan crystals. The concentration of formazan determined by the absorbance is directly proportional to number of viable cells. 1 x 10^4 cells/cm^2 cells were cultured in 96 or 48 well plates (flat bottom) for 48 hours and used experimentally thereafter. Stock solution was prepared by dissolved 50mg MTT (Sigma) in 10ml of PBS, 150µl of culture media containing 15µl of MTT stock solution was added to each well followed by incubation for 4 hours at 37ºC within their respective culturing conditions, over 6 days. A 125µl aliquot was then removed and 50µl of Dimethyl sulfoxide (DMSO) added and the plates further incubated for 45 minutes at 37ºC. As a negative control, 150µl of medium alone containing 15µl of MTT stock solution was used. The absorption was measured with a micro-plate reader (BioTek, Synergy 2) at a 570 nm wavelength.

2.6.3 Alamar blue assay

Alamar blue is an alternative approach to MTT that measures both in vitro cell proliferation and cell viability. The Alamar blue assay (Fisher Scientific) converts resazurin (non-toxic reagent) to resorufin in viable cultured cells which in turn accumulates in the medium. The cellular mechanisms of resazurin conversion in viable cells are not clear, but the role of mitochondrial reductase by generating reducing equivalents such as NADH has been suggested (Sykes et al., 2009 and Rotoli et al., 2014). A density of 1 x 10^4 cells/cm^2 were cultured in 96 well plates (flat bottom) for 48 hours and used experimentally thereafter. 100µl of Essential 8 medium containing 10µl (ready-to-use solution) of Alamar blue reagent

was added directly to each 96-well plate followed by incubation for 4 hours in three different conditions at 37^{o}C to allow cells to convert resazurin (blue color) to resorufin (pink color), and then the absorbance was measured at 570 nm wavelength. Data are presented as a mean ± standard deviation (SD) of three triplicate experiments.

2.7 DNA Methylation Analysis

2.7.1 Cell pellet preparation

The cell suspensions were generated as described in **Section 2.2.3** and centrifuged at 3000 x g for 3 minutes with 1ml culture medium, supernatant removed, and the pellet washed twice with PBS by centrifugation, before the final supernatant removed and pellets stored at -20^{o}C.

2.7.2 DNA extraction

DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. Cell pellets were thawed and re-suspended in 200µl PBS, 20 µl proteinase K and 200µl buffer AL. Contaminant RNA was eliminated by RNase A treatment (Qiagen), followed by vortexing and incubated at 56˚C in water bath for approximately 10 minutes for lysing. After incubation, 200µl of 96% ethanol was added, mixed well, and pipetted into a DNeasy spin column in a 2ml collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through was removed leaving total DNA attached to the DNeasy column membrane and then the spin column transferred into a new 2ml collection tube, washed twice, with 500µl buffer AW1 and then with 500µl Buffer AW2, by centrifugation at 8000 rpm and 13000 rpm for 1 and 3 minutes, respectively. Flow-through was discarded and the spin column placed into a new 2ml microcentrifuge tube and DNA eluted in 50µl DNase and RNase free water by centrifugation at 8000 rpm for 1 minute. This step was repeated twice

to increase the yield of extracted DNA. The integrity and concentration of DNA was determined by running 200ng DNA on 1% agarose gels using ethidium bromide staining with 1x electrophoresis buffer TAE at 100 V for 1 hour (**Figure 2.3)** and by spectrophotometrically analysis at 260 nm with a NanoDrop 2000/2000c (Thermo Scientific). Extracted DNA was stored at -20°C.

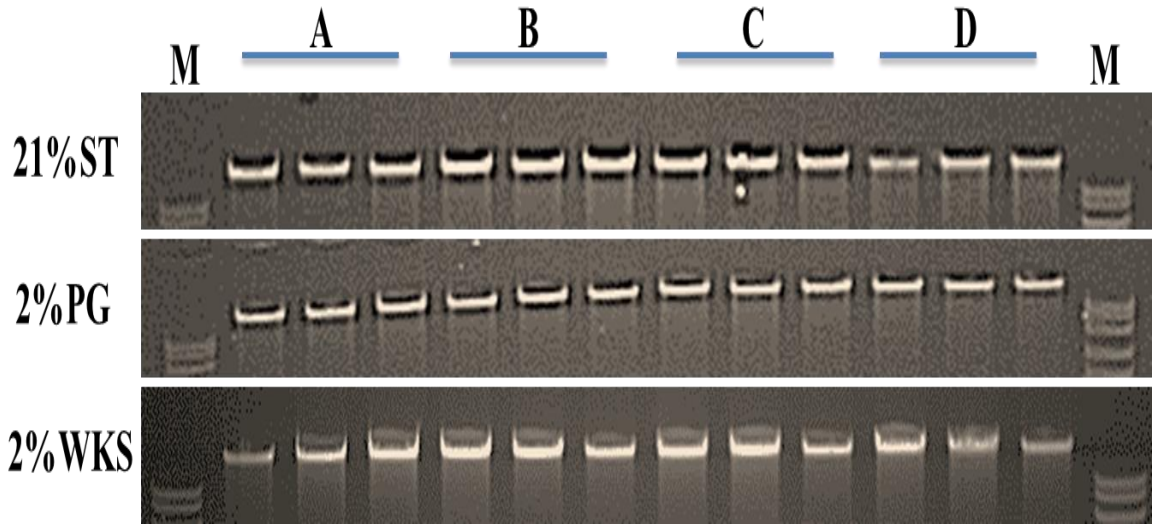


Figure 2.3 Example agarose gel electrophoresis. The quality and integrity of genomic DNA (approximately 200 ng DNA) extracted from hESCs samples incubated in normoxic (21%ST) and hypoxic (2%PG and 2%WKS) oxygen conditions using standard gel electrophoresis. A: Undifferentiated cells; B: Differentiated cells (5 Days); C: Differentiated cells (10 Days); D: Differentiated cells (20 Days); M: DNA marker. Gel images were captured with Syngene Genesnap software.

2.7.3 DNAs global methylation

2.7.3.1 Elisa-based methods

Quantification of total 5mC content within DNA isolated from cell lines was performed using the MethylFlash^{TM} methylated DNA quantification Kit (Epigentek). In brief, the DNA sample is captured in a pre-coated 96-well ELISA plate, and the methylation status detected after several incubation steps with primary and secondary antibody before quantification of

methylated DNA by reading calorimetrically at A_{450} and the quantification of global DNA methylation derived from the absorbance measured (Kurdyukov and Bullock, 2016).

2.7.3.2 5-Methylcytosine (5mC)

The ELISA analysis was performed following manufacturer's instructions; 100ng of genomic DNA for each sample (in triplicate) was used in the assay. First, a standard curve of methylated polynucleotide containing 50% of 5-methylcytosine as a positive control was generated using the five concentrations indicated into **Table 2.2**, and these were added to their respective wells.

Table 2.2 Summary of the five-different concentration of the positive control used to generate the standard curve.

Standards	Concentration of Positive control
1	0.5ng/µl
2	1.0ng/µl
3	2.0ng/µl
4	5.0ng/µl
5	10.0ng/µl

100ng of genomic DNA for each sample was quantified as previously described in **Section 2.7.2** and diluted to a final volume of 10µl with distilled water. Binding solution (80µl) was added to each well of 48 well plates. 1µl of negative control buffer (an unmethylated polynucleotide), standard and the sample DNA was added to the respective wells and mixed well by pipetted several times to ensure the solution coated the bottom of the well **(Figure 2.4)**. The plate was the sealed with a Parafilm M and incubated for 90 minutes at 37^{o}C.

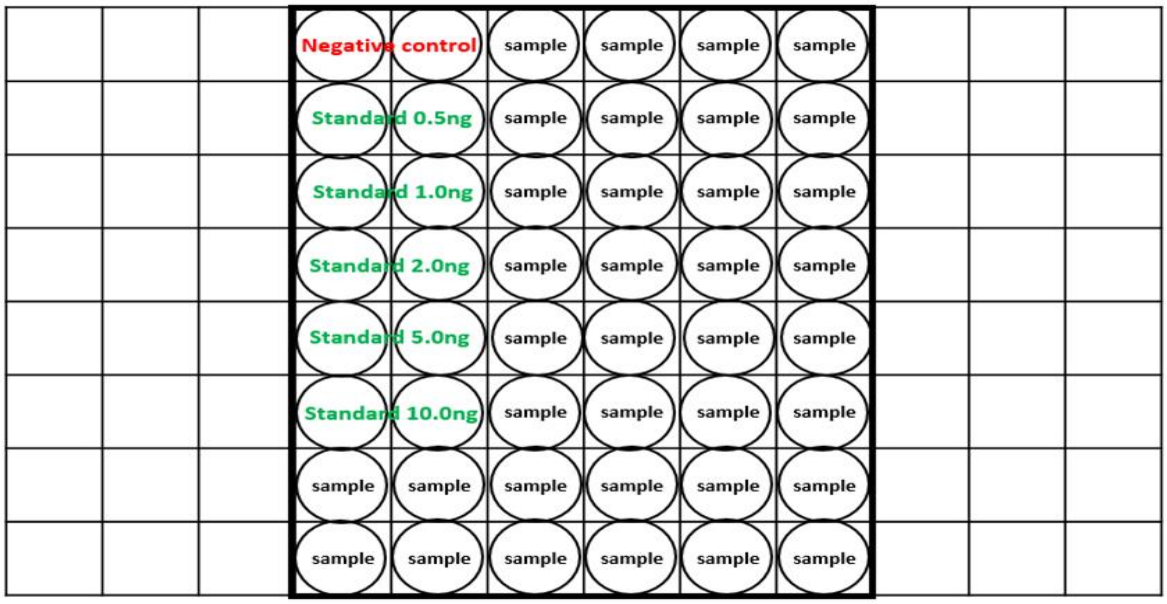


Figure 2.4 A schematic of the 48 well plate. The negative control and standards were loaded in duplicates. The samples were loaded in the remaining wells.

Then, the binding solution was removed, and the plate washed three times with 150µl of 1x wash buffer (prepared by diluted 10x wash buffer (ME1) diluted with 117ml of distilled water). Further, 50µl of a 1:1000 dilution of capture antibody was added to each well and incubated for 60 minutes at room temperature. After incubation the solution was removed before a further three washes with 150µl of wash buffer was performed again. Then 50µl of detection antibody (1:2000) was added to each well followed by incubation at room temperature for 30 minutes prior to the solution being removed and a further four washes with 150µl wash buffer. Next 50µl of enhancer buffer (diluted at a ratio of 1:5000) was added and samples incubated at room temperature for 30 minutes before removal and washed for five times, as above. Following washing, 100µl of developer solution (1:5000) was added and plates incubated at room temperature for 10 minutes in the dark. The developer solution acted as a chromogenic reagent which turned blue in the presence of sufficient methylated DNA. Following this 50µl of stop solution was added to stop the colorimetric reaction and the plate placed on a shaking frame to ensure that the reaction was completely stopped.

Absorbance was measured at 450 nm with a micro-plate reader (BioTek, Synergy 2) using the program Gen5 1.10.

Standard curve was determined using linear regression **(Figure 2.5)** and the percentage of 5mC in the total DNA calculated using the following formula:

$$5\text{-}mC\ (ng) = \frac{Sample\ OD - Negative\ control\ OD}{Slope\ x\ 2^*}$$

$$5\text{-}mC\ \% = \frac{5\text{-}mC\ Amount\ (ng)}{S}\ \mathbf{x\ 100\%}$$

Where 2* are a normalization factors in the positive control to 100%, as the positive control contains only 50% of 5mC. S is the amount of input sample DNA in ng.

2.7.3.3 5-Hydroxymethylcytosine (5hmC)

Similar to the Methylated DNA Quantification kit protocol, 200ng amount of isolated DNA was quantified using the colorimetric MethylFlash™ Hydroxymethylated DNA Quantification Kit (Epigentek). Linear regression was performed on the standard curve generated from the positive controls and the total amount **(Figure 2.5)** and percentage of methylated (5hmC) DNA quantified using the following formula:

$$5\text{-}hmC\ (ng) = \frac{Sample\ OD - Negative\ control\ OD}{Slope\ x\ 5^*}$$

$$5\text{-}hmC\ \% = \frac{5\text{-}hmC\ Amount\ (ng)}{S}\ \mathbf{x\ 100\%}$$

5* is a factor to normalize 5hmC in the positive control to 100%, as the positive control contains only 20% of 5hmC. S is the amount of input sample DNA in ng.

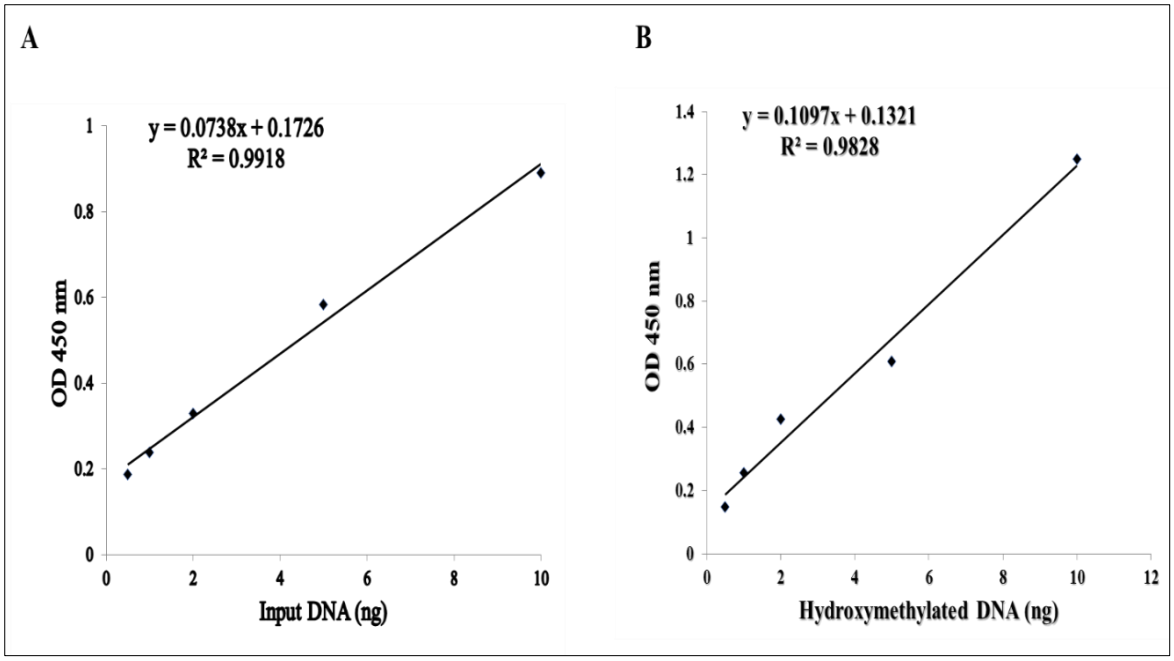


Figure 2.5 Example standard curve of determination of DNA methylation by the immunoassay. A) Linear relationship between the absorbance and amount of 5-methylcytosine. B) Linear relationship between the absorbance and amount of 5-hydroxymethylcytosine.

2.8 Gene expression analysis

2.8.1 RNA extraction

Cells were lysed with 350µl lysis buffer RLT supplemented with 10µl/ml β-mercaptoethanol to disrupt cells before being homogenized by passing the lysate through a Qiashredder spin column (Qiagen) via 2 minutes centrifugation at 17000 rpm. After homogenisation the lysate was stored at -80°C for subsequent RNA extraction. RNA was extracted followed to the manufacturer's protocol using the RNeasy® Mini Kit (Qiagen). Frozen lysates were defrosted before being placed on ice and 1 volume of 70% ethanol added, mixed well, and the resulting solution passed through an RNeasy mini spin column placed in a 2ml collection tube to allow total RNA to bind to the column membrane and centrifuged at 8000 g for 15 second before the flow through was discarded and the column membrane washed twice with

700µl of RW1 washing buffer and then with 500µl of RPE washing buffer by centrifugation at 8000 g for 15 seconds and 2 minutes, respectively. The last step was repeated by centrifuged the column again at 17,000 g for 1 minute to remove any residual fluid. Then the spin column was transferred into a new 2ml microcentrifuge tube and the membrane soaked with 25µl RNeasy-free water for two minutes prior to being centrifuged at 6000 g for 1 minute. This step was repeated to increase the RNA concentration by transferring the RNA from the microcentrifuge tube back onto the RNase spin column and centrifuged again. Total RNA concentration was quantified using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific) and stored at -80°C until required.

2.8.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.8.2.1 Primer sequences

PCR primers were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and ordered from Invitrogen Ltd while primers for TET2, HIF1A, HIF2A and pluripotent genes were obtained from ThermoFisher-Scientific. Primer sequences and product sizes are listed in **Table 2.3.**

Table 2.3 Sequence of Primers used in RT-PCR.

Primers	Sequence		Product size
DNMT1	F	5'CTCTCTCCGTTTGGTACATC 3'	**245**
	R	5'CTTTCCAAATCTTTGAGCCG 3'	
DNMT3A	F	5'AGAGCAGAGGACGAGC 3'	**192**
	R	5'ATTATCGTGGTCTTTGGAGG 3'	
DNMT3B	F	5'TAATAAGTCGAAGGTGCGTC3'	**198**
	R	5'TCTGAAGCCATTTGTTCTCG 3'	
TET1	F	5'AGCTTACCAAAATCAGGTGG3'	**221**
	R	5'GCTGCTCATCTTGAGGAATA 3'	
TET2	F	5'CCACTACCCCAACCAAAGTAACA3'	**272**
	R	5'GTAGCTCTCCACTGCTACCAAAAA3'	
TET3	F	5'CTTTATGACTTCCCTCAGCG 3'	**270**
	R	5' AGCCTTTATTTCCACCTCCT 3'	
DNMT3L	F	5'TGCTAGACAGACCCCATTTCCT 3'	**274**
	R	5'ATTGACCACTCAGGGCCATTG 3'	
HIF1A	F	5'CCACCTCTGACTTGCCTTTC 3'	**702**
	R	5'GCCGAGGGAATGGGCTTAC 3'	
HIF2A	F	5'GGCGTCTGAACGTCTCAAAG 3'	**788**
	R	5'GAGCCGCAGAAGTCTCCA 3'	
OCT-4	F	5'CATAGCCTGGGGTACCAAAATGGGG 3'	**536**
	R	5'GCAATTTGCCAAGCTCCTGAAGCAG 3'	
SOX-2	F	5'CCATCCACACTCACGCAAA 3'	**204**
	R	5'GCAAAGCTCCTACCGTACCACT 3'	
NANOG	F	5'TGCAGGACTGCAGAGATTC 3'	**300**
	R	5'GGTGGCAGAAAAACAACTGGC 3'	
β-actin	F	5' GCCACGGCTGCTTCCAGC 3'	**528**
	R	5'AGCCATGCCAATCTCATCTT 3'	

2.8.2.2 RT-PCR reaction

RT-PCR was performed on samples using the Qiagen® One step RT-PCR kit. Mix reaction was prepared according to **Table 2.4.**

Table 2.4 RT-PCR reaction mixes components

Reagents	Volume/sample
RT-PCR Buffer,5x	2.5µl
dNTP mix (10mM)	0.5µl
Forward primer (10mM)	1µl
Reverse primer (10mM)	1µl
RNase free water	3.5µl
Q-Solution, 5x	2.5µl
Template RNA (25ng)	1µl
QIAGEN One-step RT-PCR enzyme mix	0.5µl
TOTAL	**12.5µl**

Reactions were performed in a thermal cycler programmed in accordance to the conditions given in **Table 2.5**. This programme was performed for all primers except DNMT3A which had a higher annealing temperature of 56°C. A negative control (without template RNA) was used in every experiment.

Table 2.5 Cycling conditions.

Step	Time (mins)	Temperature (˚C)	Number of cycles
Reverse Transcriptase	30	50	1
Initial PCR activation	15	95	1
Denaturation	1	94	39
Annealing	1	55	39
Extension	1	72	30
Final Extension	10	72	1

2.8.2.3 Agarose gel electrophoresis

PCR products were electrophoresed through a 2% agarose gel (4 g of agarose powder was dissolved in 200ml 1x TAE buffer) containing 1:40000 ethidium bromideplaced in an electrophoresis tank containing excess 1 x TAE buffer (Sigma) that completely covered the gel. PCR samples (6µl) were mixed with a 2µl aliquot of gel loading buffer and then pipetted into the correct well. The wide range DNA marker (Sigma) was loaded into the first well and second DNA ladder placed in the final well and then visualised on the GelDoc-It2Imager (UVP, California, USA). To ensure cDNA integrity the housekeeping gene, β-actin, was also amplified. All gels were run at 100 V for one hour **(Figure 2.6)**.

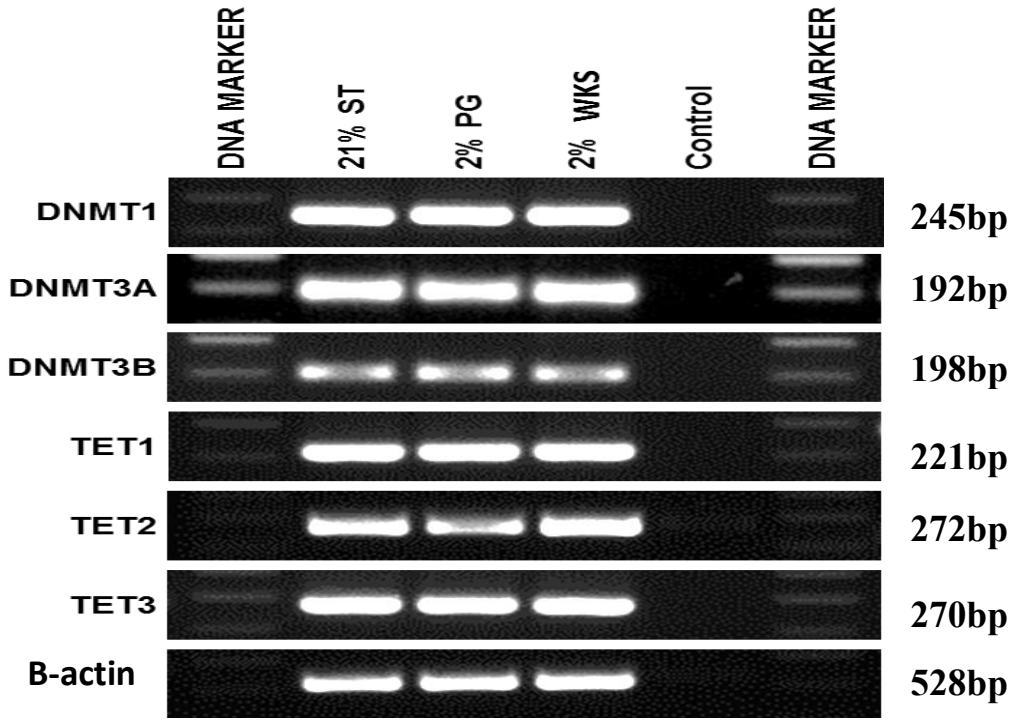


Figure 2.6 Example agarose gel electrophoresis. Agarose gel (2%) showing validation of specific PCR products of expected size for each DNMTs, TETs, and housekeeping genes tested in the present study across three different oxygen conditions. Direct load wide range DNA ladder. Fragment size (base pairs (bp)) indicated. Gel images were captured with Syngene Genesnap software.

2.8.3 Quantitative Polymerase Chain Reaction (qPCR)

Relative gene expression was performed on 25ng of isolated RNA for each sample to measure the level of expression of the genes showed in **Table 2.3**. qRT-PCR was achieved using QuantiFast® SYBER® Green RT-PCR One-step kit (QIAGEN). Relative quantification of gene expression was measured using the $2^{-\Delta\Delta CT}$ method. Threshold cycle (C_t) is the cycle number that shows a detectable increase in fluorescence generated within the PCR cycle. The expression of the genes was normalised against the house-keeping gene B-actin (Δ^{Ct}). $\Delta\Delta^{Ct}$ value was assessed as the difference between Δ^{Ct} values of the gene of interest and the control sample.

First, a qPCR reaction mix for each primer was prepared in accordance with the reaction setup indicated in **Table 2.6**.

Table 2.6 qPCR reaction mixes component

Reagents	Volume/Sample
SYBER Green Master mix	6.25µl
Forward primer	1.25µl
Reverse primer	1.25µl
QuantiFast® RT (DNA Polymerase) mix	0.125µl
Template RNA (25ng)	1.0µl
RNase free water	2.5µl
TOTAL	**12.375µl**

The reaction mix was then added to a chilled 96 PCR plate (Sigma) and sealed with optical adhesive cover (Thermofisher) before the plate was then transferred to a Strata gene MX3005P real time thermal cycler. The following cycling programme was used in accordance to the thermal profile outlined in **Table 2.7**.

Table 2.7 Thermal profile setups.

Step	Time (min)	Temperature (°C)	Number of cycles
Reverse Transcriptase	10	50	1
Initial PCR activation	5	95	1
Denaturation	10sec	95	40
Primer extension and Fluorescent determination	30sec	60	1
Pre-programmed melt curve and Fluorescent determination as temperature increased from 60^{O}C to 95^{O}C	1	95	1
	30sec	60	1
	30sec	95	1

2.9 Western Blotting

2.9.1 Preparation of cell lysates

The cells were grown as described in **Section 2.2.1**. Following two PBS washes cells were lysed with fresh lysis RIPA buffer ((150mM sodium chloride (NaCl, Sigma-Aldrich), 20mM Hepes (CalbioChem), 2mM EDTA (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 120µM leupeptin (Sigma-Aldrich), 1% NP-40 (Sigma-Aldrich), 10µM pepstatin (Sigma-Aldrich), 1mM phenylmethanesulfonylfluoride fluoride (PMSF, Sigma-Aldrich) and 10µM pepstatin (Sigma-Aldrich). The cell lysate suspension was then pipetted into an Eppendorf tube, centrifuged at 10,000 g for 10 minutes at 4°C to remove insoluble materials and the total protein content in the supernatant measured using the BCA protein kit (Sigma-Aldrich).

2.9.2 Bicinchoninic acid (BCA) protein assay

Firstly an albumin standard curve containing bovine serum albumin (BSA) was prepared at concentrations between 0.1 and 2mg/ml. BCA working reagent was prepared by mixed BCA reagent A containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N sodium hydroxide with 4% copper (II) sulphate (BCA reagent B) in the ratio of 1:50. 10µl of each BSA standard or 5µl of each lysate (diluted in 5µl RIPA buffer) was pipetted into the 96 well plate cultures and then mixed with 100µl of the working reagent. Next, the 96 well plates were covered and incubated for 30 minutes at 37°C. Absorbance readings were taken in duplicate at OD 570 nm using a spectrophotometer. Determination of the protein concentration for each lysate was performed using linear regression by plotting the BSA standard OD 570 versus its concentration **(Figure 2.7)**.

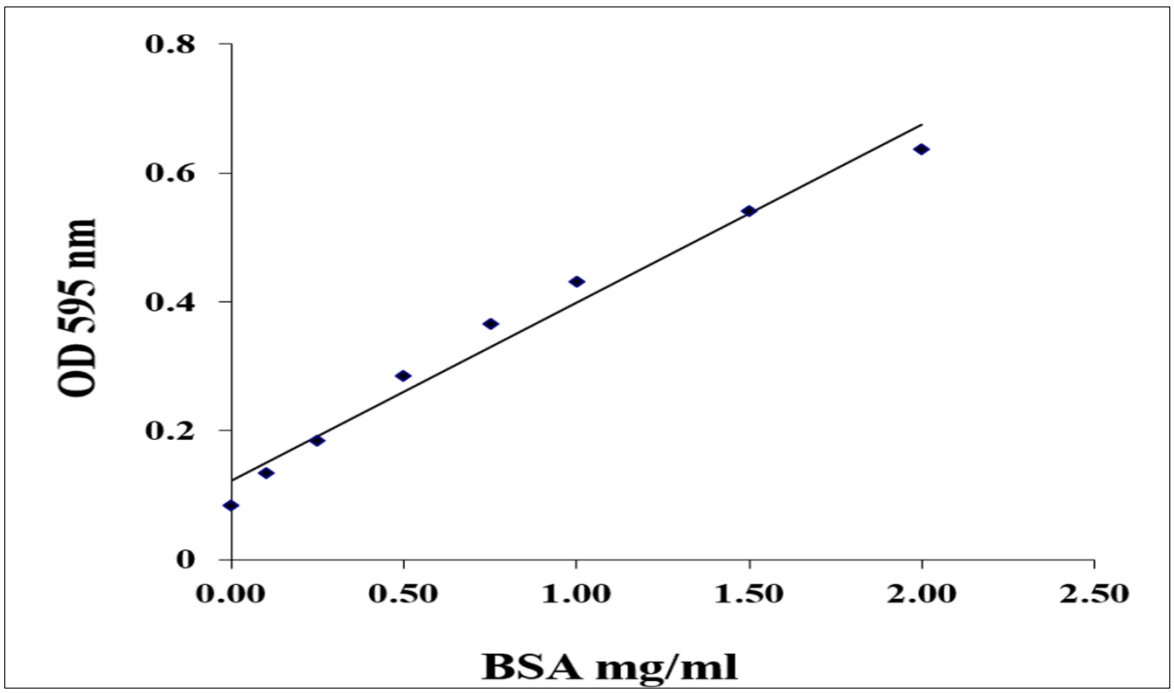


Figure 2.7 A representative standard curve of BSA protein assay. The standard curve of BCA assay using a serial dilution of BSA, the concentrations of unknown samples from cell lysate were determined from the interpolation of X-axis of the standard curve, data expressed as mean ± SD (n=3). Interpolation of x-axis was used for determination of protein concentrations of unknown samples.

2.9.3 SDS-PAGE

Samples were prepared for SDS-PAGE by added 5-15µg sample protein to NuPAGE buffer (Invitrogen) supplemented with 5% β-mercaptoethanol (Sigma-Aldrich) before denaturing at 80°C for 15 minutes. Each lane on the 4-20% NuView Tris-Glycine polyacrylamide gradient gel (Nusep) was washed by pipetting 6-8 times with hepes running buffer ((100 mM hepes, 100 mM Tris and 1% sodium dodecyl sulphate SDS (Sigma-Aldrich)) and placed in an XCell SureLock Mini Cell (Invitrogen). Hepes running buffer was also used for both the cathode and anode buffer to separate the denatured protein from each sample lysate. Sample protein was loaded to each lane on the gel and the PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) was used to estimate the size of immunodetected proteins on

each gel. The gel electrophoresis was run at 30 mA, 240 V, for 60 minutes or until the running reached the end of the gel.

2.9.4 Western Blotting analysis

Amersham Hybond P 0.45µm polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences) and filter paper (Whatmann 3 MM) was soaked with transfer buffer (25mM Tris, 200mM glycine (Sigma-Aldrich), 0.075% SDS and 10% methanol) for 20-30 minutes. After electrophoresis, separated proteins were transferred to a Hybond membrane using a blotting assembly as described (Towbin et al., 1979) and power supply was set at 25 V for 1.5 hours. Membrane blocking the was performed by incubation of the protein-blotted membrane at room temperature for 60 minutes with TBST buffer ((50mM Tris hydrochloride (Tris HCl, pH 7.4, Sigma-Aldrich), 150mM NaCl, and 0.1% Tween 20)) supplemented with 5% skimmed milk powder. The membrane was incubated in 5ml of diluting primary antibody at 4°C overnight with gentle rocking. Any unbound, excess antibody was removed by washing the membrane 3 times with TBST buffer and then incubating with 5ml of secondary antibody at room temperature for 1 hour with gentle rocking. Following on from washes the immunoreactivity bands were subjected to UpLight HRP chemiluminescent substrate solution (Uptima) and imagined using a FluorChem M Imager. Bands were quantified on ImageJ2 software when necessary. The antibodies and corresponding dilutions used are described in **Table 2.8**.

Table 2.8 List of human primary and secondary antibodies.

Primary antibody	Host	Company/ Catalog N.	Dilution	Secondary antibody	Company/ Catalog N.	Dilution
DNMT3B	Mouse	R&D System/ MAB7646	2 mg/ml	Anti-mouse IgG-HRP	Cell Signaling/ 70765	1:1000
TET1	Mouse	ThermoFisher/ GT1462	1:1000	Anti-mouse IgG-HRP	Cell Signaling/ 70765	1:1000
HIF1A	Rabbit	Novusbio/ NB100-479	1:1000	Anti-rabbit IgG-HRP	Cell Signaling/ 70745	1:1000
HIF2A	Rabbit	Novusbio/ NB100-122	1:750	Anti-rabbit IgG-HRP	Cell Signaling/ 70745	1:1000
GAPDH	Mouse	Merck/ MAB374	1:5000	Anti-rabbit IgG-HRP	Cell Signaling/ 70765	1:1000

2.10 Pyrosequencing

Pyrosequencing is a sequence-based technology that can be used for measuring the level of DNA methylation across many CpG sites within genes of interest. The estimated of methylation status within the sequenced region of interest are built on the signal intensities for incorporated dGTP and dATT, which release visible light through enzymatic conversion. The amount of light produced is directly proportional to the number of bases incorporated and is detected by CCD sensors within the Pyrosequencing hardware. The quantitative results are generated as percentage values from the ratio of methylated to unmethylated cytosine residues within CpG region. In this study we used the bisulphite conversion and

standard pyrosequencing protocols to determine the methylation status of genes of interest. There are many steps in pyrosequencing assay as shown in **Figure 2.8** and the details are described below.

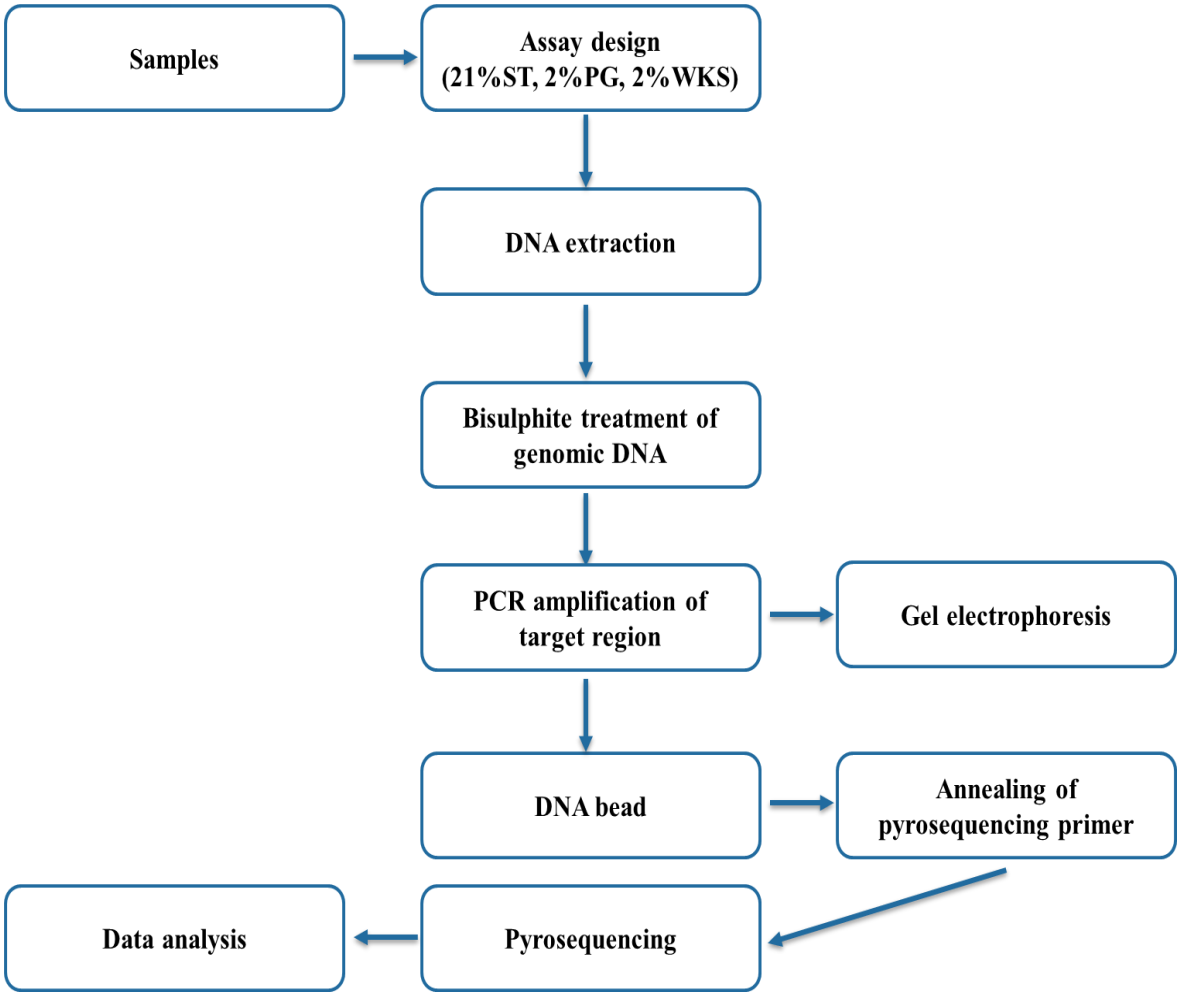


Figure 2.8 An overview of the procedure for the pyrosequencing assay for DNA methylation analysis.

2.10.1 Sodium Bisulphite Modification of Genomic DNA

Modification of genomic DNA was performed with EZ DNA Methylation-Gold™ Kit (Zymo Research) to convert unmethylated cytosine residues chemically to uracil and leave the methylated cytosine unchanged. DNA was extracted from cultured cells as described in

Section 2.7.2, and 500ng of gDNA for each sample was diluted in 20µl of DNases/RNase free water. Then 130µl of CT Conversion reagent was added to 20µl of the DNA dilution, mixed well, placed in a PCR tube and the thermal cycler run at 98^{o}C for 10 minutes and thereafter 64^{o}C for 2.5 hours. Samples were then incubated at 4^{o}C for 20 hours before being mixed with 600µl M-Binding Buffer and transferred to a Zymo-Spin^{MT} IC Column and centrifuged at 13000 g for 30 seconds. M-Binding buffer was removed by washing with 100µl wash buffer and then 200µl M-Desulphonation Buffer added followed by incubation at room temperature for 15-20 minutes. Following on from centrifugation at 13000 g for 30 seconds, 200µl washing buffer was added to remove desulphonation buffer and samples then centrifuged at 13000 g for 1 minute before being repeated twice, and the eluate was discarded. Next, the spin columns were transferred to 1.5ml microcentrifuge tubes and eluted in 21µl M-Elution Buffer centrifugation at 13000 g for 30 seconds. The sodium-bisulphite converted DNA was quantified by spectrophotometry (single-stranded DNA measurements ssDNA) and stored at -20^{o}C in sterile tubes for future use.

2.10.2 PCR Amplification

Converted DNA (2-4µl) was used as template in PCR reactions. Primers were designed from the UCSC genome browser (http://genome.ucsc.edu/) and ordered from biomers.net GmbH (Germany). Forward or reverse primers were biotinylated at the 5'end to enable sequencing of the antisense strand of the PCR amplicon. Primer sequences and expected PCR product size are listed in **Table 2.9**.

Table 2.9 Pyrosequencing PCR primers. Forward and reverse primer sequences used for pyrosequencing are shown with their respective sequencing primers. The expected products size as well as the optimum annealing temperatures degree for each primer is also given.

Gene name	Annealing Temp (°C)	Forward primer	Reverse primer	Sequencing primer	Product size
DNMT1	61	5'-TGGGGGAGGAATTAAA-3'	5′-AACTTTCTCCCCACACC-3′	5′-TTGTGTAGAAATGGAA-3′	230
DNMT3A	61	5′-GGAGAGGGTGAGAAGG-3′	5′-TATCCCCCCCCCAATT-3′	5′-GTTTGTTGTTAGGAGT-3′	196
DNMT3B	61	5′-GGGGATAGGAGAAGGG-3′	5′-TCCTCACCTCCCTTAA-3′	5′-GTTTGTTGTTAGGAGT-3′	80
DNMT3L	60	5′-GGAGAGGAATAGAGTG-3′	5′-AAAAATAACCCCTTCT-3′	5′-CCTCCATTCCAATACA-3′	72
TET1	61	5′-GGGTTTAGGTTGTGG-3′	5′-CCCCAACTCCACCTAC-3′	5′-TTTAAAGTTGTGGGGAT-3′	81
TET2	61	5′-TGGTGAGGGGTGTTAG-3′	5′-CCCTTTAAAATACACT-3′	5′-CACTCCACCACCACT-3′	150
TET3	61	5′-TTGGGATAGGGGTTAG-3′	5′-CCACTCCCTAATCTAT-3′	5′-GATAGGGGGGGTTAGT-3′	88

GoTaq®G2 Flexi DNA Polymerase kit (Promega) was used to amplify all samples in 25µl volumes containing 5µl 5x Flexi buffer, 0.2µl Taq DNA Polymerase, 2.5µl of 25mM MgCl2, 1µl 20µ M/µl nucleotides, 1µl of each forward and reverse primer and 12.3µl molecular

grade water. Initial denaturation was performed with one cycle of 95°C for 5 minutes. Then, touch-down cycling was used for the first 14 cycles, where the temperature was reduced by 0.5ºC in each successive cycle. Next, 35 cycles of 95°C for 45 seconds, annealing at 55–63°C for 45 seconds and elongation at 72°C for 30 seconds. followed by final elongation step with one cycle at 72°C for 5 minutes. Successful PCR amplification was electrophoresed through a 2% agarose gel as described in **Section 2.8.2.3.**

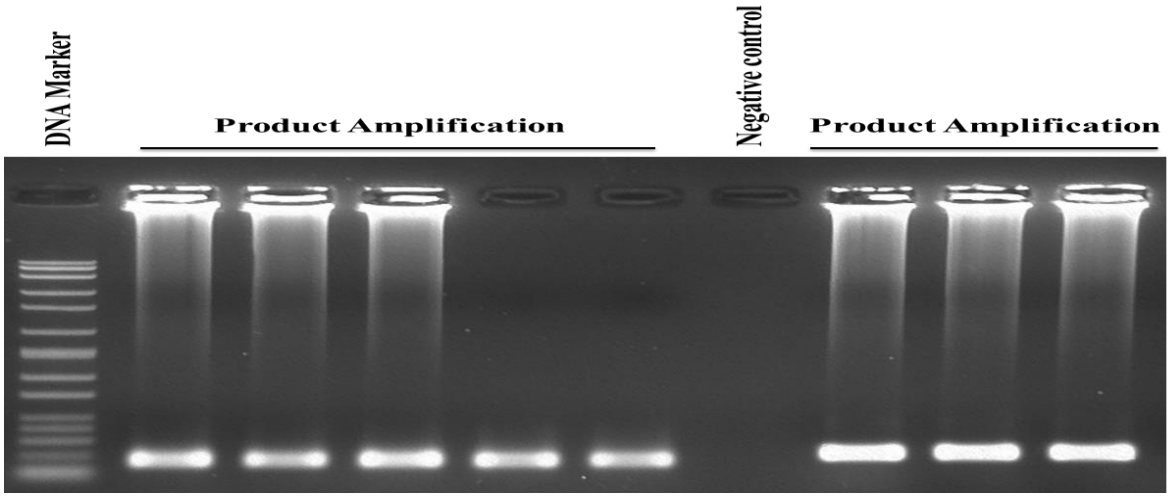


Figure 2.9 PCR amplification products by electrophoresis on a 2% agarose gel. The products are directed toward bisulphite-converted DNA, and specific for the DNMT3L gene. The successful and specific amplification are suggested when the correct size and single band are presence. Negative control: no-template.

2.10.3 Pyrosequencing

PCR amplied DNA was used in pyrosequencing analysis to determine the percentage of methylation within genes of interest. PCR products were biotin–labelled, by mixing PCR products with 1µl of streptavidin-sepharose beads (GE Healthcare), 40µl of Binding buffer (Qiagen), and 19 µl of molecular grade water, and 40µl of Binding buffer (Qiagen) on a rotary shaker for 15 minutes to allow streptavidin beads to bind biotin-labelled strand. During the mixing step, the Q24 pyrosequencing plate was prepared by adding 25 µl of

Annealing buffer (Qiagen), and 0.08µl of the assay-specific sequencing oligonucleotide to each well **(Table 2. 9)**.

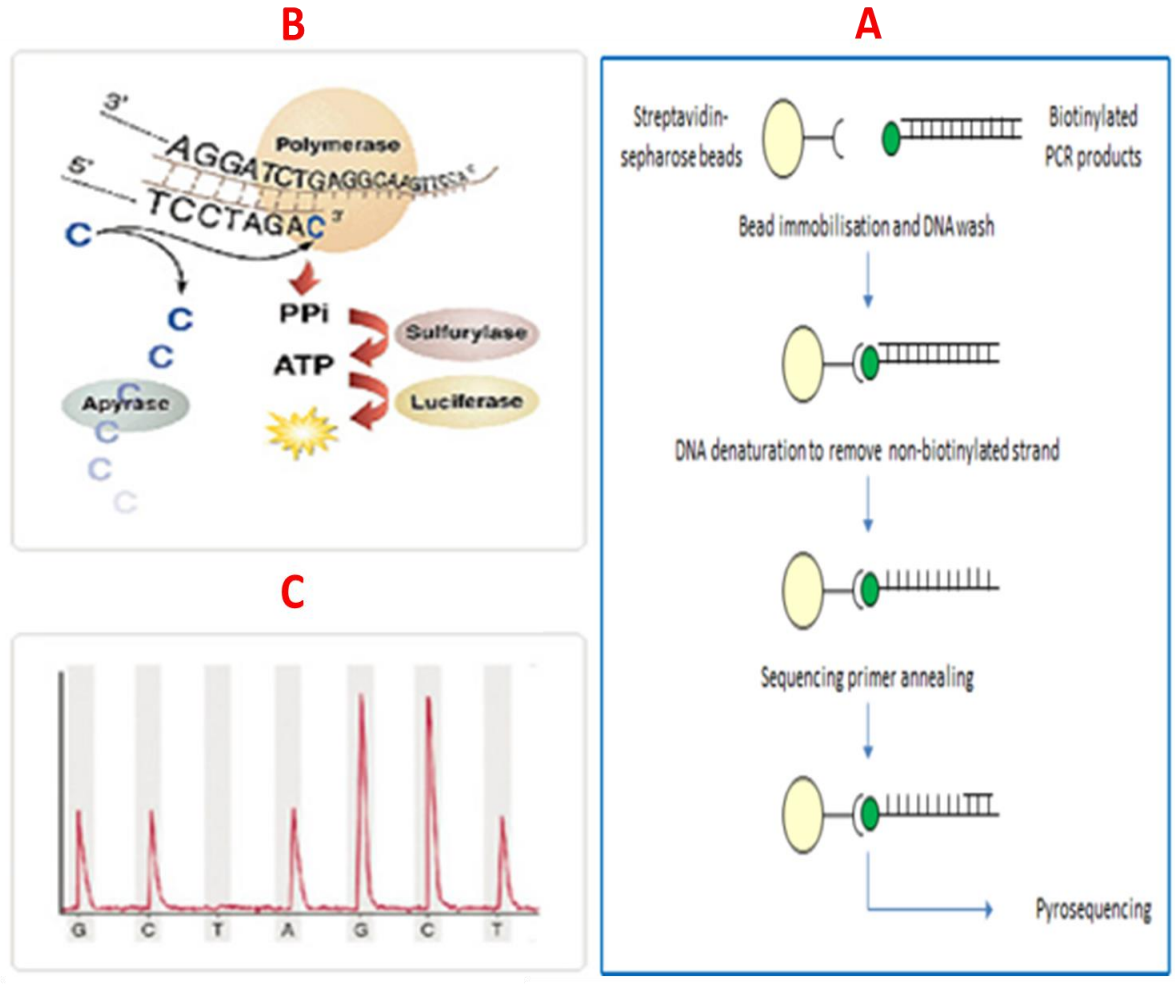


Figure 2.10 An overview of principles of pyrosequencing reaction steps. A) gDNA is bisulphite converted, PCR amplified with one of the primers biotinylated and "captured" by the streptavidin-sepharose beads. The beads do not pass through the vacuum station filters and are preserved during the washing, the biotinylated strand is purified and annealed to a sequencing primer to yield only the target region of interest before they subject to Pyrosequencing. B) This figure shows the chemical reactions and enzymes involved in the producer of light. The incorporation of complementary base-pairs into the single-stranded template is catalysed by the polymerase enzyme within the provided enzyme mixture. Pyrophosphate (PPi) is released during this chemical reaction and converted to ATP by sulfurylase enzyme in the presence of adenosine 5´-phosphosulfate substrate (APS). ATP is a required to convert luciferin to oxyluciferin that is catalysed by luciferase enzyme, and as sequence results the light is produced. the apyrase enzyme removes any unincorporated nucleotides. C) Detection is built on the intensity of the light produced and then the ratio of

cytosines to thymines can be determined by using the Pyrosequencing software. This figure adapted from the Invitrogen Pyrosequencing™ guide.

Samples were then removed from the shaker and transferred to the Pyromark Q24 Vacuum Workstation (Qiagen), cleaned with 70% ethanol to remove excess salts and solutes, denatured with NaOH solution (VWR) to leave the bead-bound strand of interest only, and purified with wash buffer (10mM Tris pH 7.6) to remove the unbiotinylated DNA strand of interest before the biotinylated strands was released into the Q24 pyrosequencing plate filled with annealing mix. The plate was heated at 80^{o}C for 2 minutes on preheated heat blocks and then cooled for 3 minutes to allow annealing between the sequencing primer and the target DNA strands. Pyromark Gold Q24 reagents (Qiagen) including the four nucleotides and the substrate and enzyme mix were loaded into pyrosequencing dispensation cartridge. Volumes were determined with the PyroMark Q24 software and control samples with each assay confirmed consistency between plates. The sequencing data was analysed using the PyroMark software.

2.11 Statistical analysis

The experimental data were analysed using statistical software SPSS (IBM SPSS Statistics 21). A comparison among three groups was assessed using a one-way analysis of variance (ANOVA). The threshold for which statistically significant was accepted as $p<0.05$. GraphPad Prism 5 (GraphPad Software, La Jolla, USA) was used for graphical displaying of data. Data are expressed as mean ± SD, each result represent a replicate of 3 independent experiments (n=3).

Chapter 3: Air oxygen culture of human bone marrow-derived mesenchymal stem cells drives DNA hypermethylation

3.1 Introduction

Human bone marrow-derived mesenchymal stem cells (BM-hMSCs) are characterized by their multipotency and differentiation ability (Schwartz et al., 2002). Human bone marrow-derived mesenchymal stem cells (BM-hMSCs) were the first MSCs isolated and since then they have been reported from a wide range of adult tissues, such as dental tissues, adipocytes, lung, muscle, and peripheral blood (Mimeault and Batra, 2006). Moreover, and due to the multipotency potential (osteoblast, adipocyte and chondrocyte) and immunosuppressive properties MSCs are widely used in many clinical applications and evaluated as a potential tool of stem cells for regenerative medicine (Wang et al., 2016). However, short term survival, early senescence, poor engraftment following transplantation, and genetic and epigenetic instabilities during *ex vivo* expansion are among the weaknesses of MSC-based regenerative therapies (Mastri et al., 2014). It has been established that a complex mixture of inter- and intracellular interactive signalling systems are involved in control of homeostasis, growth, and differentiation of MSCs in their niche (Haque et al., 2013; Kowalski et al., 2015). Among which, physiological normoxia is involved in many aspects such as regulation of stem cell behaviour, maintenance of stemness and metabolism (Mohyeldin et al., 2010; Narva et al., 2013). The prevalent oxygen level of the BM-hMSCs niche has been shown to be 1-2% O_2, suggesting that physiological normoxia is a predominant characteristic (Park et al., 2010). Currently, increased consideration has been given to cell culture conditions and how this could alter data collection *in vitro*. Several studies have suggested that reduced oxygen culture conditions, reminiscent of physiology, (2–5% O_2 concentration) enhance isolation and proliferation of BM-hMSCs with less accompanying DNA damage and genetic instability (Boregowda et al., 2012; Estrada et al. 2012; Haque et al., 2013; Heywood and Lee, 2017). Notably, starkly reduced oxygen levels play a significant role in maintaining stem cell fate by regulating stability of the hypoxia-

inducible factor (HIFs) transcription factors which in turn stimulate different genes (Hu et al., 2016; Wiafa et al., 2018). However, the precise role of low, physiological, oxygen conditions on stem cell biology are still under investigation (Simon and Keith, 2008).

"Epigenetics is the study of heritable changes in gene expression that are not caused by changes in the DNA sequence" (Esteller, 2008; D'Urso and Brickner, 2014). There are many mechanisms by which epigenetics can control the process of gene expression such as DNA methylation and histone modification (Bird, 2007). However, many studies have suggested a strong link between DNA methylation and regulation of stem cell renewal and differentiation (Huang et al., 2013). In addition, DNA methylation has been shown to have different patterns in BM-hMSCs in aging and in long-term cultures, suggesting that DNA methylation is involved in regulation of replicative senescence and aging (Bork et al., 2010). Lineage-specific promoter hypermethylation results in a loss of BM-hMSCs ability to differentiation towards that lineage. However, the correlation between changes on CpG island methylation levels of BM-hMSCs and ability of these cells to differentiate during long-term culture is still not clear and further work is needed (Sørensen et al., 2010). The work of Shahrzad *et al* reports that low oxygen levels significantly influence changes in DNA methylation and result in a global reduction in DNA methylation in human colorectal and melanoma cell lines (Shahrzad et al., 2007). Also, it has suggested that reduced oxygen plays a role in the regulation of DNMT and TET expression through induction of the HIF pathway and this can catalyse changes in the levels of 5mC and 5hmC in a number of human cell lines (Wu et al., 2015; Chen et al., 2016). However, the role of physiological normoxia conditions in regulation of the level of 5mC and 5hmC and their enzymes (DNMT and TET, respectively) in BM-hMSCs have received very little attention.

We have tested the hypothesis that epigenetic methylation marks (5mC and 5hmC) in human bone marrow-derived mesenchymal stem cells (BM-hMSCs) are altered in response to

specific environmental changes (physiological normoxia (2% O_2) vs. air oxygen (21% O_2)). Furthermore, we have investigated if these changes are in relation to changes in the expression of controlling DNMTs/TETs gene expression and translation. This was achieved by isolation and expanded hMSCs from human bone marrow aspirates by plastic adherent culture technique and by examining global patterns of 5mC and 5hmC and examining local patterns within the promoter regions of DNMTs/TETs genes.

3.2 Methods

3.2.1 Isolation and culture of human bone marrow-derived mesenchymal stem cells (BM-hMSCs)

Three human mesenchymal stem cells (MSCs) (BMA-16, BMA-20 and BMA-25) were isolated and expanded from commercially sourced **(Table 3.1)** whole human bone marrow aspirate (BMA) using the plastic adherence method as described in **Section 2.4**. The cells were maintained in the following three conditions 21% O2 - Standard cell culture (21%ST); 2% O2 - Pre-gassed media – 2% O_2 incubation (2%PG) and 2% O2-Pre-gassed media, Workstation (2%WKS). To maintain the oxygen level of the cells during physiological oxygen culture, cell culture media was de-oxygenated to a predefined oxygen concentration (2% O2) as described in **Section 2.2.2**.

Table 3.1 Donor details of human bone marrow aspirates

No	BMA label	Donor description	Supplier
1	BMA-16	Human Bone Marrow, Male, Age-29 years	Lonza, MD, USA
2	BMA-20	Human Bone Marrow, Female, Age-50 years	AllCells, LLC, SA
3	BMA-25	Human Bone Marrow, Male, Age-27 years	Lonza, MD, USA

3.2.2 Characterisation of mesenchymal stem cells

hMSCs were seeded at 2 x 10^4 cells/cm^2 in 24 well plates in complete culture media prior to being cultured in differentiation media for 20 days. Evaluation of differentiation potential of BM-hMSCs was as described in **Section 2.4.1.1** and **Section 2.4.1.2**. Flow cytometry of differentiated hMSCs was as described in **Section 2.4.1.3**.

3.2.3 Global 5mC and 5hmC analysis

Total genomic DNA was isolated from hMSCs as described in **Section 2.7.2**, and 100ng and 200ng of DNA was subjected to methylated DNA (5mC) and 5-hydroxymethylated DNA (5hmC) quantification assay (Colorimetric) respectively by using a MethylFlash quantification kit as outlined in **Section 2.7.3**.

3.2.4 Quantitative real-time RT-PCR

Total RNA was extracted from hMSCs as described in **Section 2.8.1**. Relative gene expression was carried out in triplicate samples by using the QuantiFast SYBR Green OneStep RT-PCR kit **(Section 2.8.3)**.

3.2.5 Protein analysis

hMSCs were cultured and lysed as described in **Section 2.9.1** before 30µg of total protein **(Section 2.9.2)** was subjected to Western Blot analysis using antibodies against DNMT3B, TET1, HIF1A and HIF2A as described in **Chapter 2, Section 2.9.4**.

3.2.6 Pyrosequencing of sodium bisulphite-converted DNA

Genomic DNA (500ng) was subjected to bisulphite conversion before pyrosequencing using a PyroMark Gold Q24 Reagents and PyroMark Q24 Software 2.0 **(Full details in Chapter 2, Section 2.10)**.

3.3 Results

3.3.1 Characterisation of Bone Marrow-derived Mesenchymal Stem Cells (BM-hMSCs)

3.3.1.1 Differentiation of BM-hMSCs into mesenchymal lineage

Confirmation of the ability of BM-hMSCs to undergo tri-lineage differentiation is essential to evidence a multipotent differentiation capacity allowing us to refer to these cells as stem cells. As mentioned in **Section 2.4.1.1**, hMSCs were exposed to either chondrogenic, adipogenic, or osteogenic differentiation media. As shown in **Figure 3.1**, hMSCs were positive for Alizarin Red staining for osteogenesis, Oil Red O staining for adipogenesis, and Alcian blue staining for chondrogenesis. Cells grown in control media stained weakly, if at all, with the respective tri-lineage differentiation dyes **(Figure 3.1)**.

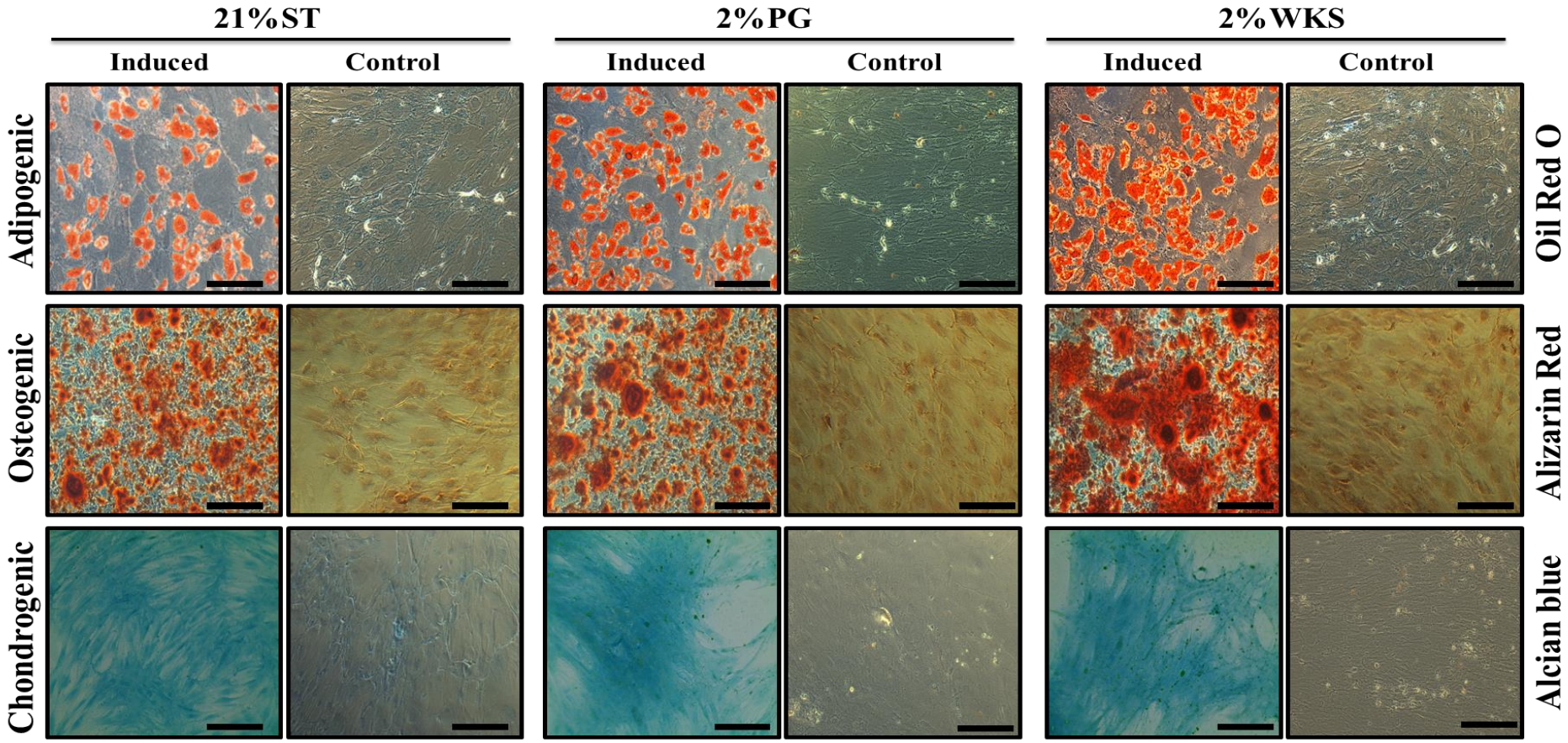
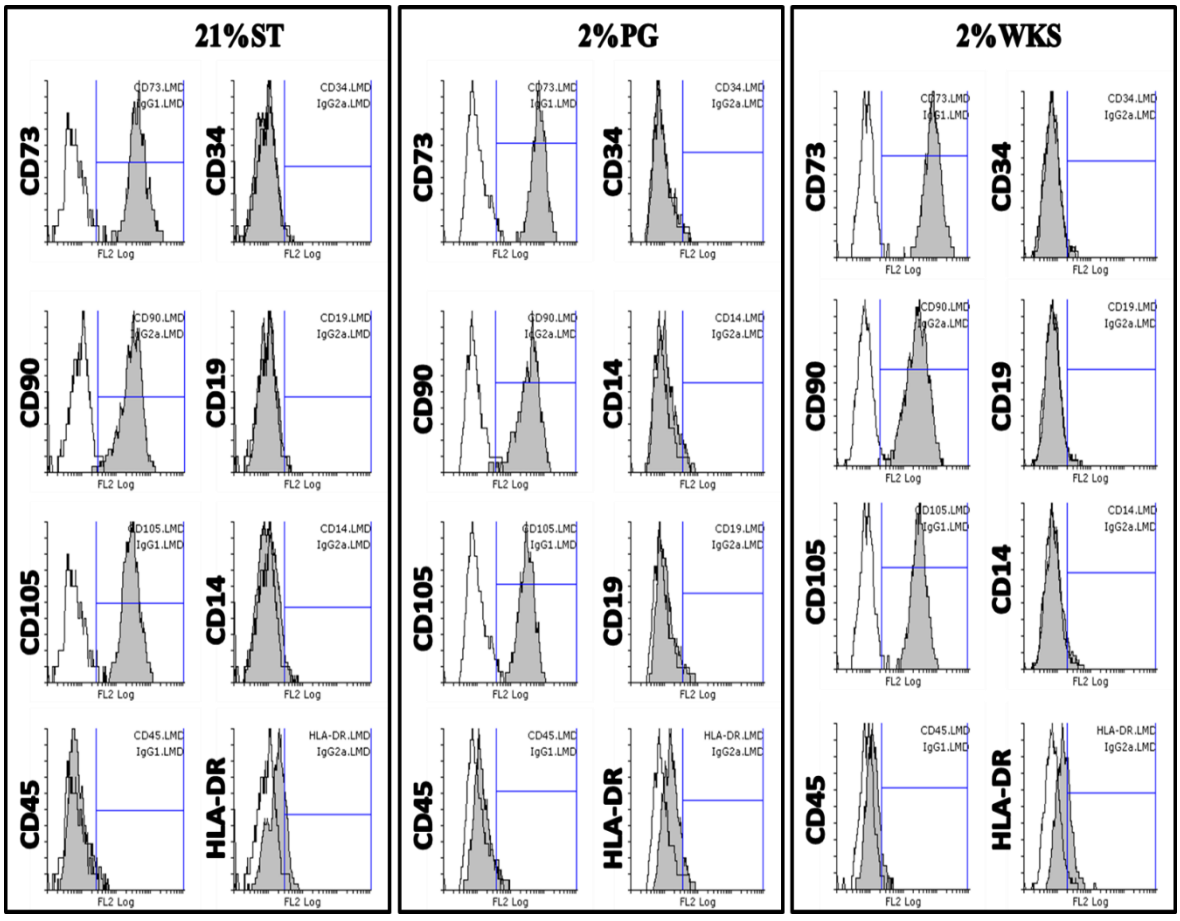


Figure 3.1 Classical Tri-lineage differentiation of hMSC cultured in air oxygen (21%ST) and physioxia (2%PG and 2%WKS). hMSC were seeded into Tri-lineage differentiation medium for 20 days, revealing adipocytes (Oil Red O-lipid droplet), osteoblasts (Alizarin Red-calcium deposits) and chondrocytes (Alcian blue–proteoglycans stain). The scale bar equal 100 μm for all images.

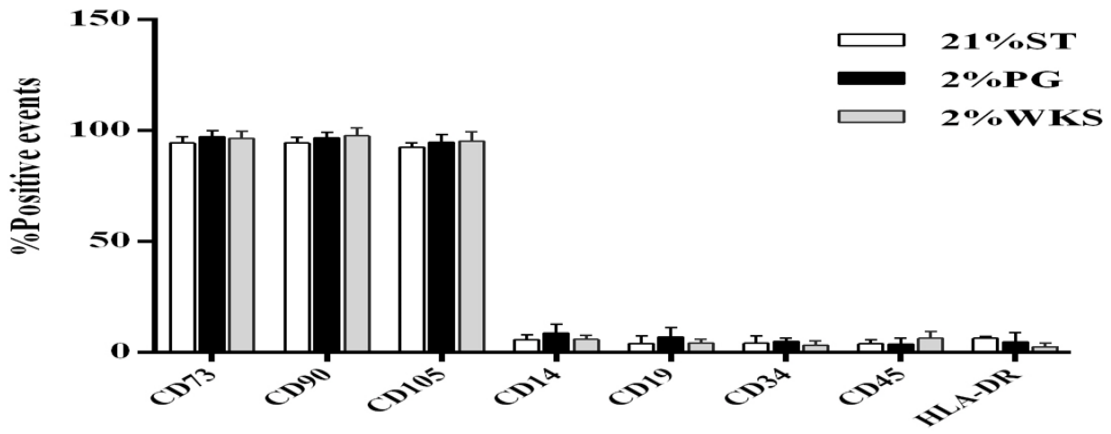
3.3.1.2 Immunophenotypic characterisation of BM-hMSCs by flow cytometry

According to ISCT guidelines (Dominici et al., 2006), a panel of hMSC surface markers were used to determine the immunophenotype of hMSCs by FACS. Cells from each condition (21%ST, 2%PG and 2%WKS) were incubated with marker antibodies and the percentage of positive or negative events were determined relative to the relevant isotype control marker (IgG1 or IgG2a) **(Figure 3.2A)**. The results indicated that hMSCs expressed CD90, CD73 and CD105 but lacked expression of CD14, CD19, CD45, CD34 and HLA-DR surface markers. No significant differences between isolation conditions were observed **(Figure 3.2B).**

(A)



(B)



21%ST	94.3	94.4	92.3	5.8	4	4	3.8	6.4
2%PG	97.1	96.5	94.5	8.5	7	4.9	3.6	4.6
2%WKS	96.4	97.6	95.6	5.8	4.2	3.2	6	2.5

Figure 3.2 Immunophenotypic characterisation of BM- hMSCs. A) Overlay histograms of hMSCs by Flowing Software showed that the hMSCs in three oxygen conditions expressed were typically negative for (CD14, CD19, CD37, CD45, HLA-DR) and typically positive for (CD90, CD73 and CD105). The unfilled area represents isotype control IgG1 or IgG2a, grey area is antibody marker. B) The percentage positive event was quantified relative to the relevant isotype control marker; IgG1 (CD73, CD90, CD105, and CD19) or IgG2 (CD14, CD34, CD45, and HLA-DR). Y-axis indicates surface markers. X-axis indicates % of positive events. The values are mean (n=3) and the error bar indicate standard deviation (SD).

3.3.2 Physiological normoxia induced expression of pluripotency markers in BM-hMSCs

Reduced oxygen culture resulted in significantly enhanced mRNA levels of the pluripotency marker SOX-2 but no significance indicated for OCT-4 (POU5F1) or NANOG. The relative fold expression of SOX-2 was significantly increased in 2%WKS cultures compared to air oxygen 21%ST in BMA-16, BMA-20 and BMA-25 (2.0 ± 0.29, 2.3 ± 0.44 and 2.0 ± 0.54, respectively), and in 2%PG cultures compared to air oxygen 21%ST in BMA-20 and BMA-

25 (2.2 ± 0.54 and 1. 8 ± 0.44, respectively) **(Figure 3.3)**. OCT-4 expression was generally higher in low oxygen than air oxygen but was not significantly upregulated. In contrast, the level of NANOG was decreased in hMSCs following treatment with 2%PG and 2%WKS but no significant differences were noticed. The data from all three BM-MSCs (BMA-16, 20 and 25) was merged to have as representative biological replicates and to avoid cell line-specific biases as possible. Similar signficant increase was observed in SOX-2 level in cells cultured under 2%PG and 2%WKS (1.8 ± 0.36 and 2.1 ± 0.18, respectively) **(Figure 3.3)**. The results also showed there was no significant change in the relative expression of OCT-4 and NANOG in hMSCs cultured under reduced oxygen compared to air oxygen 21%ST **(Figure 3.3).**

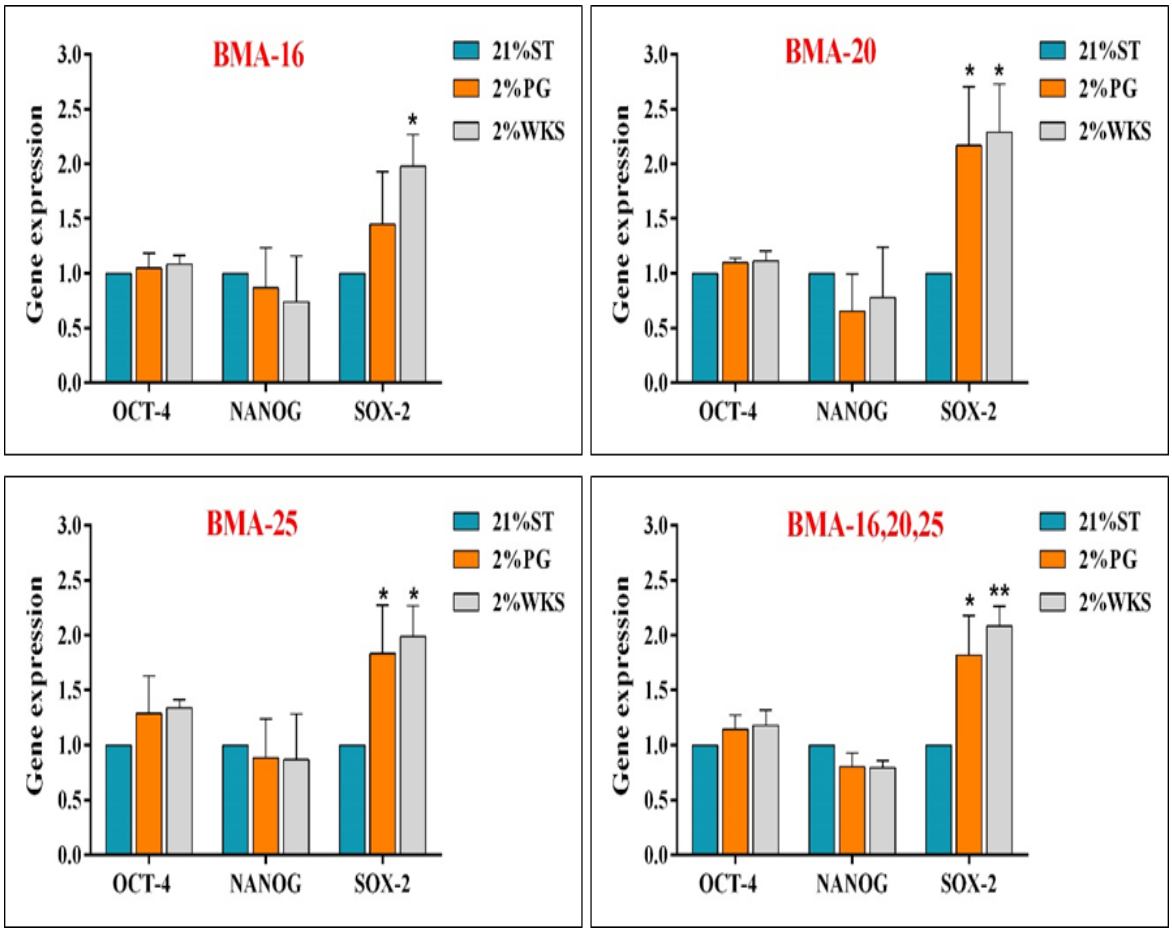


Figure 3.3 Quantitative RT-PCR analysis of OCT-4 (POU5F1), NANOG, and SOX-2 in response to reduce oxygen in three BM-hMSCs. Expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to untreated

cell. X-axis indicates pluripotency markers Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

3.3.3 Air oxygen induces DNA hypermethylation (5mC and 5hmC) in BM- hMSCs

To examine the global DNA methylation (5mC and 5hmC) patterns in BM- hMSCs DNA was isolated from hMSCs (Passage1) after isolation and 21 days incubation in 21%ST, 2%PG and 2%WKS. This DNA was then explored using an immunospecific method to determine global levels of 5mC and 5hmC. A reduced level of global DNA methylation in the total DNA extracted from all three hMSCs cultured in both reduced oxygen conditions (2%PG and 2%WKS) in comparison to oxygen air (21%ST) was noted **(Figure 3.4 and 3.5)**.

The level of 5mC in BMA-16 cells was significantly lower following incubation in 2%PG (0.67 ± 0.17, p<0.05) and 2%WKS (0.54 ± 0.08, p<0.01) in comparison to those cultured in 21%ST (0.99 ± 0.10) **(Figure 3.4A)**. Also, as shown in **Figure 3.4A** DNA methylation decreased significantly in BMA-20 in 2%PG, by a percentage change of 5-methylcytosine (0.36 ± 0.05, p<0.05), and in 2%WKS (0.25 ± 0.05, p<0.01) versus 21%ST (0.61 ± 0.015). BMA-25 cells cultured in 2%WKS showed significant reduction in 5mC content (0.37 ± 0.06) in comparison to those cultured in 2%PG and 21%ST (0.64 ± 0.16 and 0.85 ± 0.06), respectively **(Figure 3.4A)**. Also, the results of three BM-MSCs (BMA-16, 20 and 25) taken together showed significant reduction in global 5mC level in cells cultured under 2%WKS conditions (0.39 ± 0.15, p<0.05), but there were no significant differences was observed between the cells that cultured in 2%PG and 21%ST (0.56 ± 0.17 and 0.82 ± 0.2) **(Figure 3.4B)**.

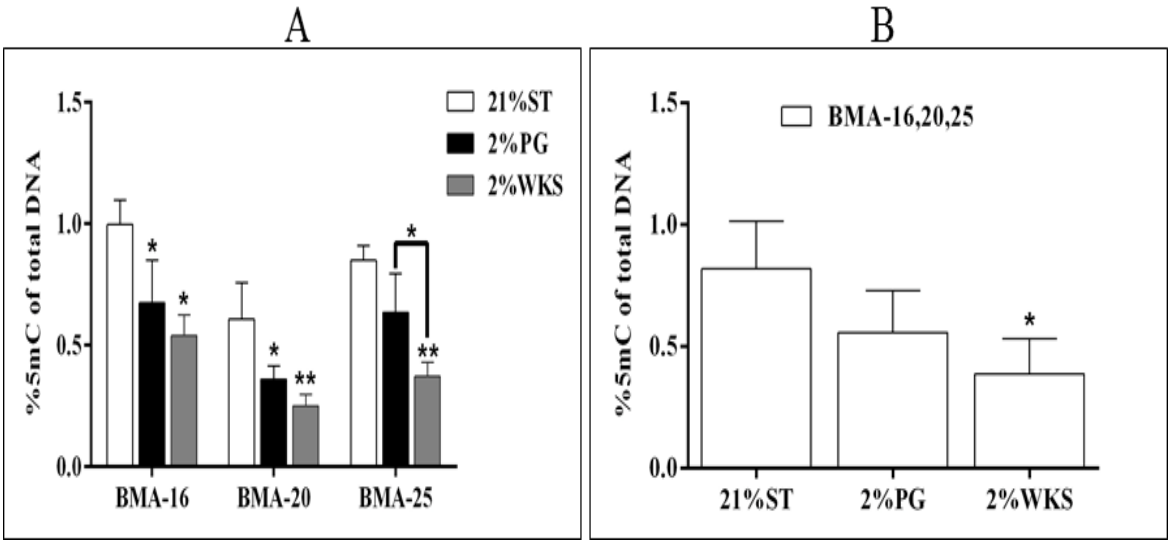


Figure 3.4 Global changes of 5mC contents in BM- hMSCs. A) Three BM- hMSC lines were incubated in air oxygen (21%ST) and normoxia physiological conditions (2%PG and 2%WKS). B) The results of three BM-MSCs (BMA-16, 20 and 25) taken together. The methylated DNA fraction in the total isolated DNA was measured using MethylFlashTM Methylated DNA quantification kit. Y-axis indicates 5-methylcytosine absorbance (450 nm). X-axis indicates different oxygen culture conditions. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Similar significant reductions were observed in global 5hmC content in BMA-16 cells grown under 2%WKS condition 0.009 ± 0.002, $p<0.05$ in comparison to those cultured in 2%PG and 21%ST (0.16 ± 0.0036 and 0.016 ± 0.0008), respectively **(Figure 3.5A)**. The level of 5hmC in BMA-20 cells was decreased significantly following incubation in 2%WKS (0.009 ± 0.0015, $p<0.01$) in comparison to those cultured in 21%ST (0.017 ± 0.002) **(Figure 3.5A)**. Also, as shown in **Figure 3.5A** 5hmC decreased in cells of BMA-25 grown in reduced oxygen versus 21%ST, but there were no significant differences was observed. Also, the results of three BM-MSCs (BMA-16, 20 and 25) taken together showed significant reduction in global 5hmC level in cells cultured under 2%WKS condition (0.009 ± 0.0007, $p<0.05$) versus 21%ST (0.019 ± 0.004) **(Figure 3.5B)**.

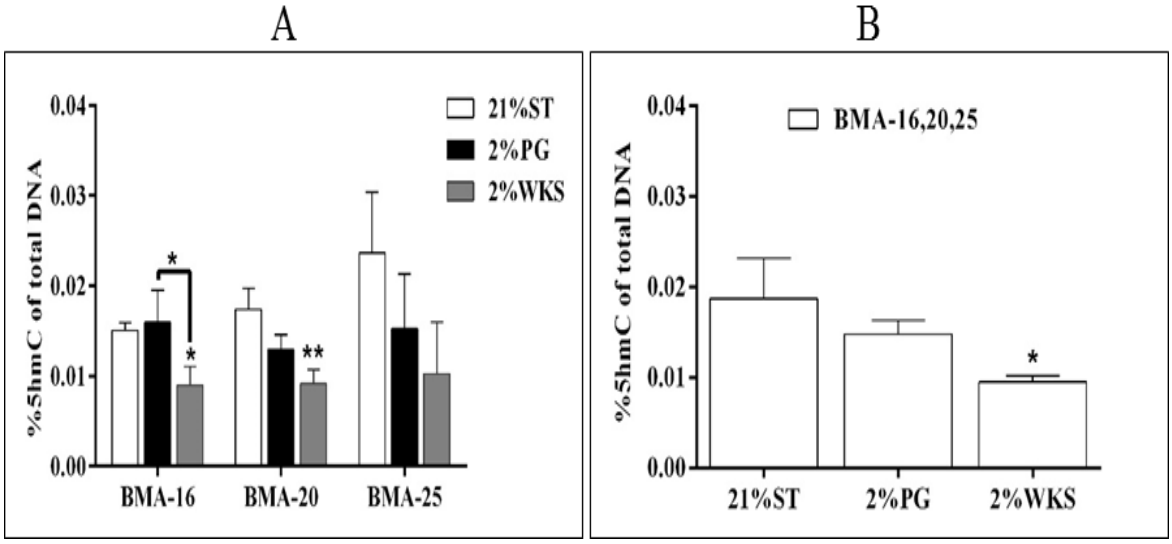


Figure 3.5 Global changes of 5hmC contents in BM- hMSCs. A) Three BM- hMSCs were incubated in air oxygen (21%ST) and normoxia physiological conditions (2%PG and 2%WKS). B) The results of three BM-MSCs (BMA-16, 20 and 25) taken together. The methylated DNA fraction in the total isolated DNA was measured using MethylFlash^{TM} hydroxymethylated DNA quantification kit. Y-axis indicates 5-hydroxymethylation absorbance (450 nm). X-axis indicates different oxygen culture conditions. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

3.3.4 Expression changes of DNMTs and TETs genes by BM- hMSCs in response to reduced oxygen

RT-qPCR was employed to examine whether the reduced levels of global DNA methylation (5mC and 5hmC) in response to physiological normoxia were in relation to changes in the expression of DNMTs/TETs genes. The results showed there was no significant change in the relative expression of DNMT3A in all three BM-hMSCs **(Figure 3.6)**. The level of DNMT3B was decreased significantly in BMA-16 and 20 cells following treatment with 2%WKS (0.43 and 0.38, P<0.01) respectively, and in BMA-16 and 25 cultured in 2%PG (0.57 and 0.6, P<0.05, respectively) versus 21%ST **(Figure 3.6)**.The level of DNMT3B was decreased significantly in BMA-16 and 20 cells following treatment with 2%WKS (0.43 and

0.38, P<0.01) respectively, and in BMA-16 and 25 cultured in 2%PG (0.57 and 0.6, P<0.05, respectively) versus 21%ST **(Figure 3.6)**.

Similar significant reductions were observed in DNMT3L levels BMA-16 cells grown under 2%PG and 2%WKS conditions (0.76, P<0.01 and 0.69, P<0.05), respectively versus 21%ST **(Figure 3.6)**. Significant differences were also noted in expression levels of DNMT3L between BMA-20 cells cultured in 2%PG and 2%WKS (1.5 and 0.71, P<0.05) respectively. In addition, the level of DNMT1 was significantly higher in BMA-16 cells cultured in 2%WKS (1.4, P<0.05) versus 21%ST.

The results of three BM-MSCs (BMA-16, 20 and 25) taken together showed significantly less expression in DNMT3B in cells cultured under 2%PG and 2%WKS conditions (0.64 and 0.59, P<0.01), respectively versus 21%ST, but there was no significant change in the relative expression of DNMT1, DNMT3A and DNMT3L **(Figure 3.6)**.

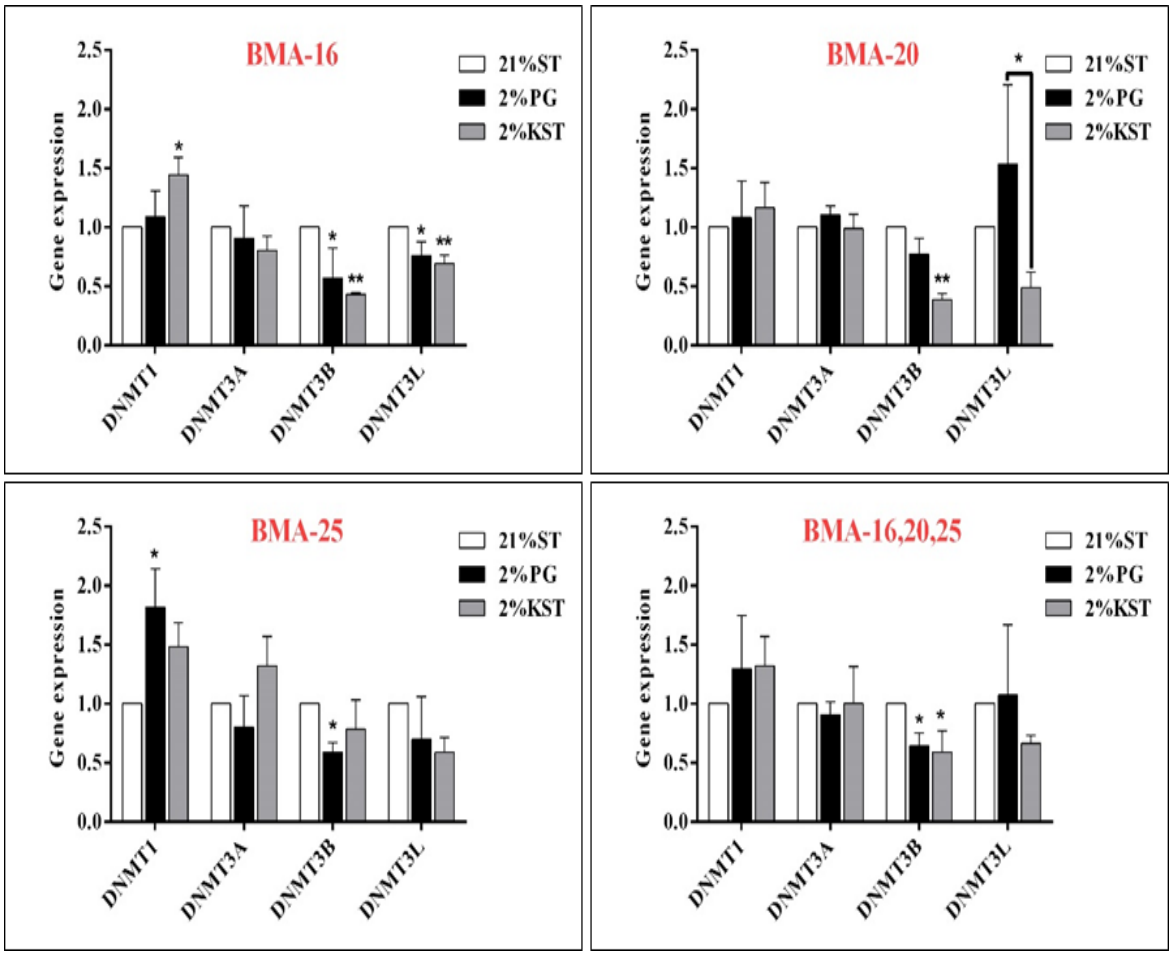


Figure 3.6 RT-qPCR expression of the DNA methyltransferase (DNMTs) enzymes at RNA level in three BM-hMSCs. Quantification RNA of DNMTs isolated from BM-hMSCs following incubation in air oxygen (21%ST) and normoxia physiological culturing conditions (2%PG and 2%WKS). The expression was normalized to the expression of β-actin. Y-axis represents relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to untreated cell. X-axis represents DNA methyltransferase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Ten-eleven translocation methylcytosine dioxygenase 2 (TET2) displayed no significant changes in expression across in all three of the BM-hMSCs **(Figure 3.7)**. TET1 expression was significantly reduced in BMA-16 cells in 2%PG and 2%WKS culture (0.7, P<0.05 and 0.64, P<0.01), respectively, and in BMA-20 cultured in both reduced oxygen (2%PG and 2%WKS) (0.48 and 0.39, P<0.01), respectively versus 21%ST **(Figure 3.7)**. Similarly, significantly less expression of TET1 in BMA-25 cells grown under 2%PG (0.6, P<0.05)

versus 21%ST **(Figure 3.7)**. Also, significant reductions in expression of TET3 in BMA-20 cells cultured in 2%PG (0.62, P<0.05) were noted. In contrast, BMA-25 showed significantly greater levels of TET3 expression in cells cultured under 2%PG condition (1.5, P<0.05) versus 21%ST **(Figure 3.7)**. In order to have as representative biological replicates and to avoid cell line-specific bias, the results of three BM-MSCs (BMA-16, 20 and 25) was merged together **(Figure 3.7)**. The results showed significantly less TET1 expression in cells cultured under 2%PG and 2%WKS conditions (0.7 and 0.77, P<0.01), respectively versus 21%ST, but there was no significant change in the relative expression of either TET2 or TET3 **(Figure 3.7)**.

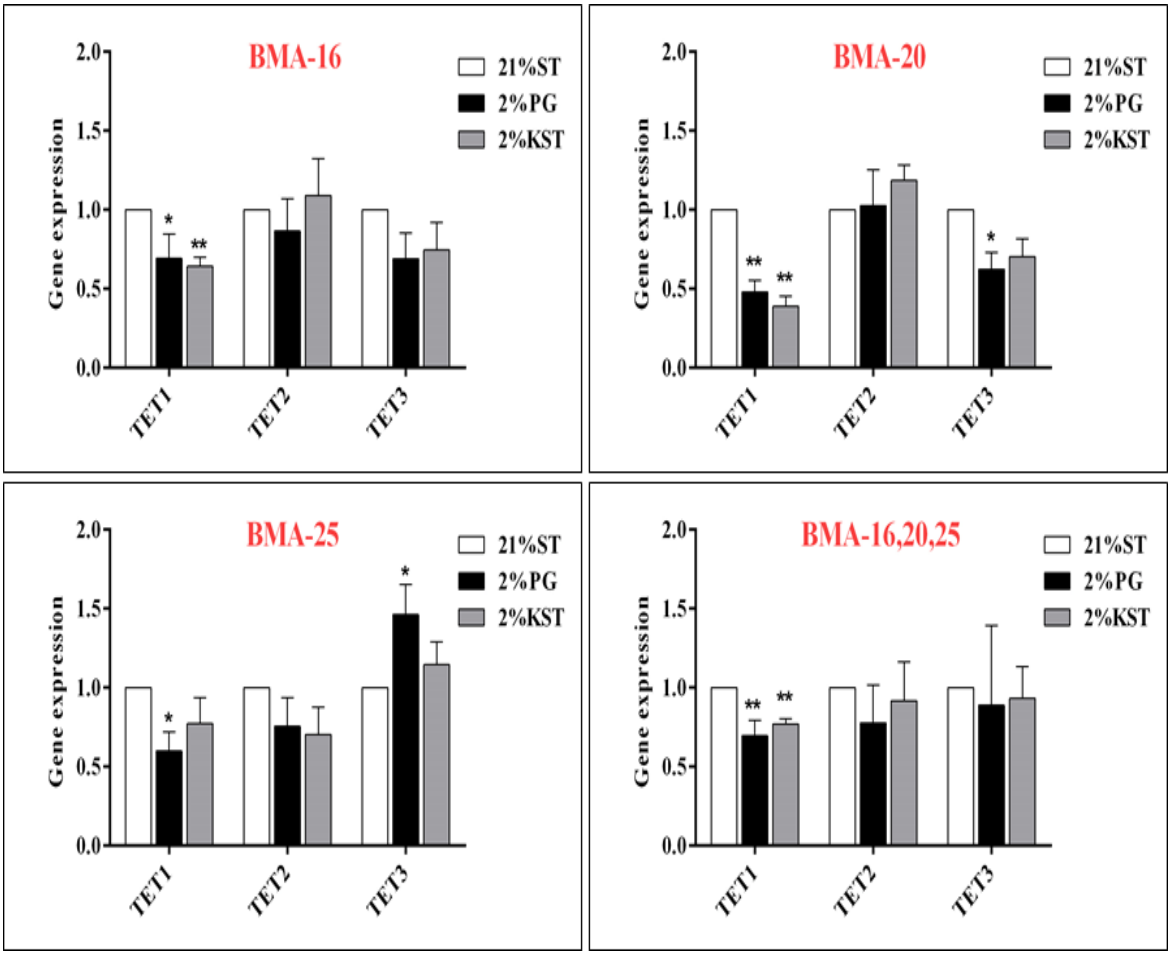


Figure 3.7 RT-qPCR expression of the ten-eleven translocation methylcytosine dioxygenase (TETs) enzymes at RNA level in three BM-hMSCs. Quantification RNA of TETs isolated from BM- hMSCs following incubation in air oxygen (21%ST) and normoxia physiological culturing conditions (2%PG and 2%WKS). The expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to

untreated cell. X-axis indicates ten-eleven translocation methylcytosine dioxygenase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

3.3.5 Reduced DNMT3B and TET1 protein expression in BM-hMSCs cultured in physiological normoxia

To confirm that the decrease in DNMT3B and TET1 transcription was accompanied by down-regulation of DNMT3B and TET1 at protein level we used Western Blot analysis on protein isolated from three BM-hMSCs cultured at day 21 in normoxia physiological conditions compared to cells maintained in air oxygen conditions. Profiling of protein expression showed that there was a downregulation of DNMT3B and TET1 in hMSCs cultured in reduced oxygen conditions in a broadly similar pattern to that observed with mRNA expression **(Figure 3.8)**.

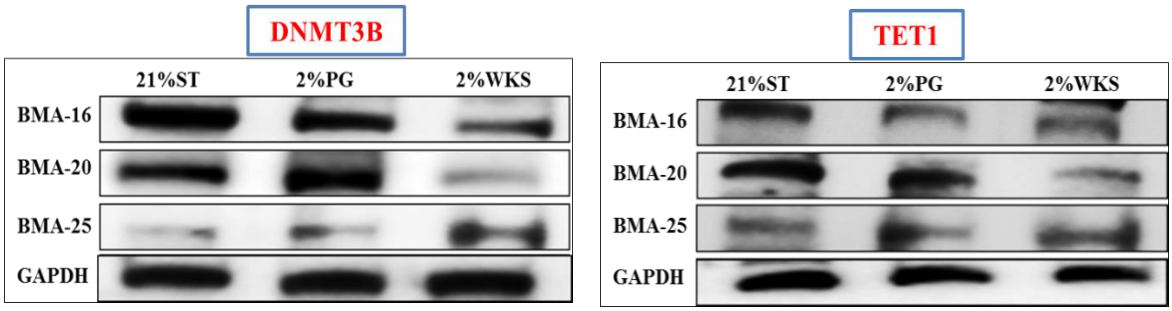


Figure 3.8 Changes in DNMT3B and TET1 expression in BM-hMSCs, at protein levels measured by immunoblotting. Protein was isolated from three BM-hMSCs at day 21 following incubation in air oxygen (21%ST) and normoxia physiological (2%PG and 2%WKS) conditions. GAPDH was used as a control.

3.3.6 Oxygen induces changes in gene-specific promoter methylation in BM-hMSCs

Section 3.3.3 showed that there were differences in the mean levels of DNA methylation between reduced oxygen and air oxygen conditions in BM-hMSCs. We also showed that low oxygen induced changes in BM-hMSCs of methylation machinery at both transcript and protein levels **(Section 3.3.4 and 5)**. To confirm that these changes were consistent with methylation at promoter level, the DNA methylation status of seven gene promoters at the CpG level was determined using pyrosequencing **(Section 2.10)**. The results showed there was significantly greater mean levels of DNMT3B promoter methylation in BMA-16 cells following isolation with 2%WKS (43%) when compared with air oxygen (13%) **(Figure 3.9)**. Similar results were observed in BMA-20 cultured in 2%PG and 2%WKS (38% and 36%, respectively) when compared with the air oxygen (7%), and in BMA-25 cells following treatment with 2%PG and 2%WKS (42% and 46%) respectively, versus 21%ST (13%) **(Figure 3.9)**. The combined analysis of three BM-hMSCs showed significantly greater DNMT3B promoter methylation levels in cells cultured under 2%PG and 2%WKS conditions (34% and 42%), respectively versus 21%ST (11%), but there was no significant change in the relative promoter methylation levels of DNMT1, DNMT3A and DNMT3L **(Figure 3.9)**.

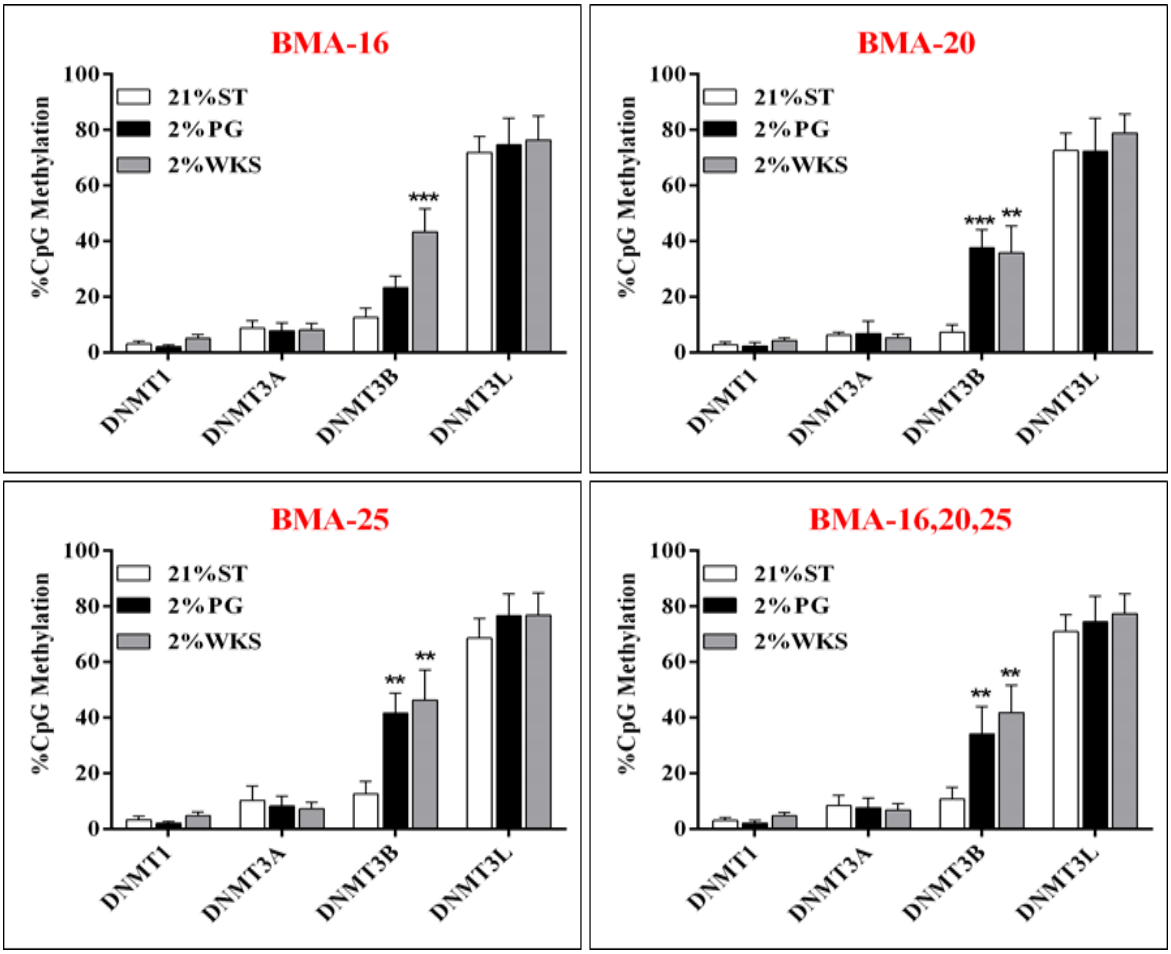


Figure 3.9 Mean levels of promoter methylation in physiological normoxia relative to air oxygen in three BM-hMSCs. A set of DNMTs genes promoter were evaluated using pyrosequencing. Y-axis represents DNA methylation levels at CpG regions. X-axis represents DNA methyltransferase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01, ***P<0.001 vs air oxygen (21%ST).

In addition, a significant increase in TET1 promoter methylation was noticed in BMA-16 and BMA-20 cells cultured in 2%PG (13% and 20%, respectively) when compared to air oxygen (6% and 11%) **(Figure 3.10)**. There was also a significant difference in the mean level of DNMT3B promoter methylation between 2%PG and 2%WKS in BMA-16 (5% and 27%), respectively **(Figure 3.10)**. In contrast, a significant increase in TET3 promoter methylation level was observed in BMA-25 cells cultured in 2%WKS (20%) versus 21%ST (4%) **(Figure 3.10)**. No significant change in the methylation level of TET2 promoter was

seen across all three BM- hMSCs. Furthermore, when taken together we saw no significant change in the methylation levels of TET1, TET2 and TET3 **(Figure 3.10)**.

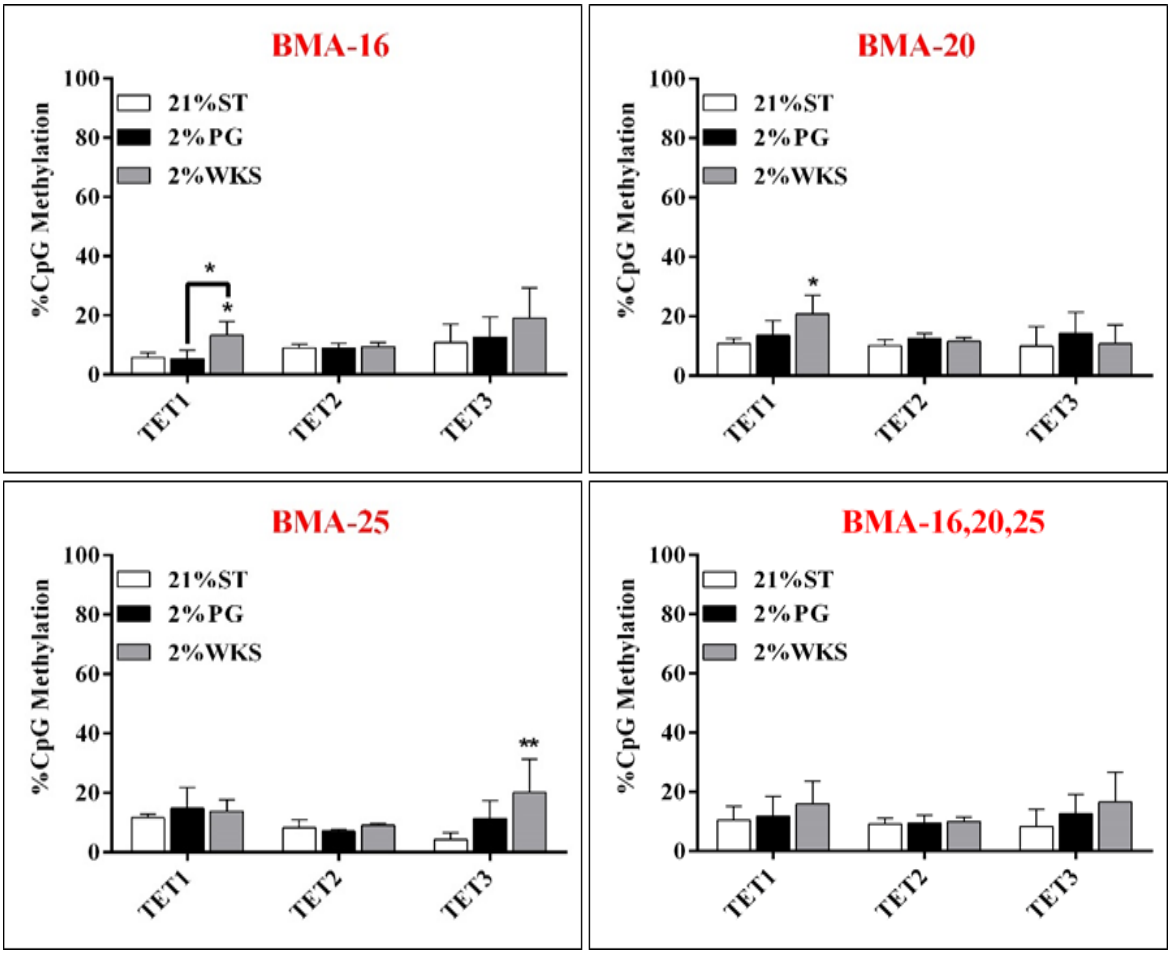


Figure 3.10 Mean levels of promoter methylation in physiological normoxia relative to air oxygen in three BM-hMSCs. A set of TETs genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates ten-eleven translocation methylcytosine dioxygenase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

3.3.7 Reduced oxygen resulted in enriched HIF2A expression at protein level in BM-hMSCs

To identify the effects of reduced oxygen incubation on the HIFs expression profiles, transcriptional and protein levels of HIF1A and HIF2A in all three BM-hMSCs cultured in

different oxygen conditions were analysed with qPCR and Western blotting assays. The results showed there was significantly less relative gene expression of HIF1A (0.76 ± 0.11 and 0.76 ± 0.10), respectively, in BMA-16 cells cultured in 2%PG and 2%WKS conditions when compared to those cultured in air oxygen (21%ST). In addition, the level of HIF2A (2.2 ± 0.54) was significantly elevated in BMA-16 cells following treatment with 2%PG **(Figure 3.11A)**. Similarly, the level of HIF2A protein (2.4 ± 0.54 and 2.2 ± 0.43) was increased significantly in BMA-16 cells cultured under 2%PG and 2%WKS when compared with air oxygen. HIF1A protein was not detected in BMA-16 cells **(Figure 3.11B**).

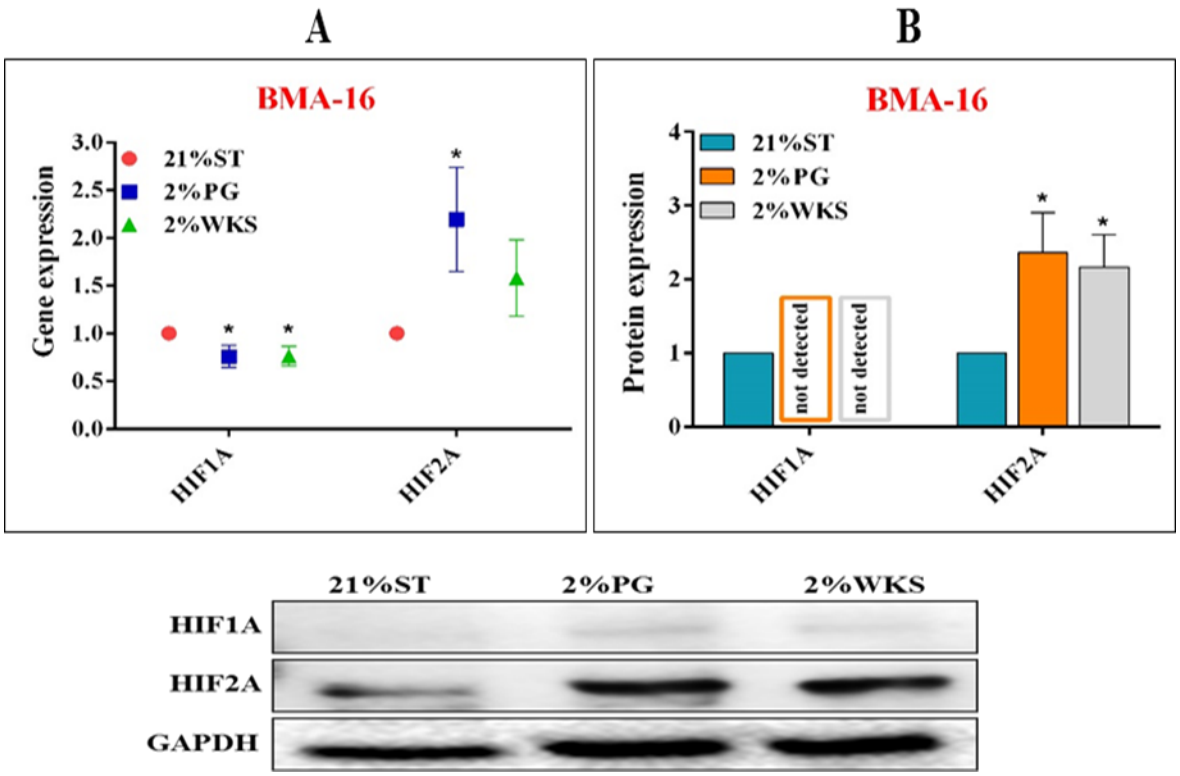


Figure 3.11 HIFs expression in BMA-16 cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Similarly, significantly less HIF1A gene expression (0.59 ± 0.09) was noted in BMA-20 cells grown under 2%WKS condition when compared with the air oxygen **(Figure 3.12A)**. In contrast, the level of HIF2A (2.04 ± 0.54) was increased significantly in BMA-25 cells cultured under 2%WKS. HIF2A protein (2.8 ± 0.7) was increased significantly in BMA-20 cells cultured under 2%WKS when compared with air oxygen. HIF1A protein was not detected in BMA-20 cells **(Figure 3.12B)**.

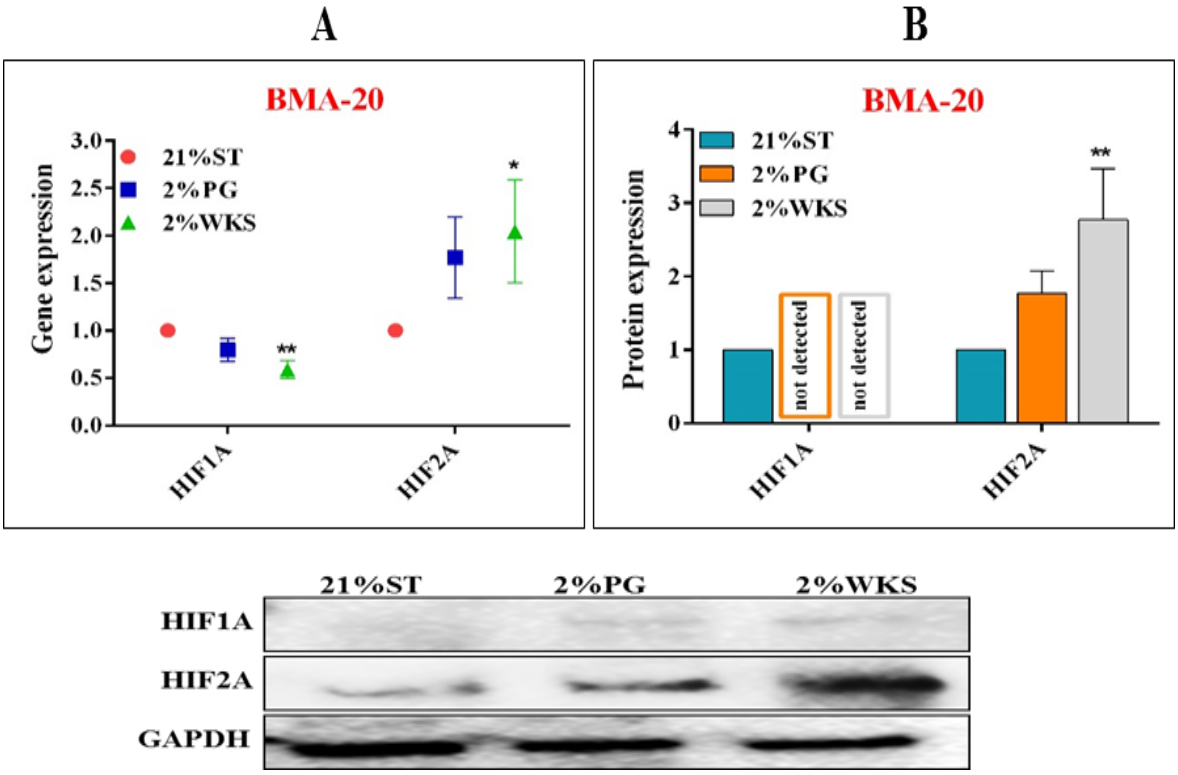


Figure 3.12 HIFs expression in BMA-20 cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

We noted significantly less relative gene expression of HIF1A (0.66 ± 0.18 and 0.59 ± 0.15), respectively in BMA-25 cells cultured in 2%PG and 2%WKS conditions in comparison to those cultured in air oxygen (21%ST). No significant changes in the gene expression levels

of HIF2A was noticed in BMA-25 cells (Figure 3.13A). A significant increase in HIF2A protein expression was noted in 2%PG and 2%WKS conditions in BMA-25 (2.2 ± 0.41 and 2.1 ± 0.38) respectively, when compared with air oxygen **(Figure 3.13B)**. No significant differences in the level of HIF1A protein were noted.

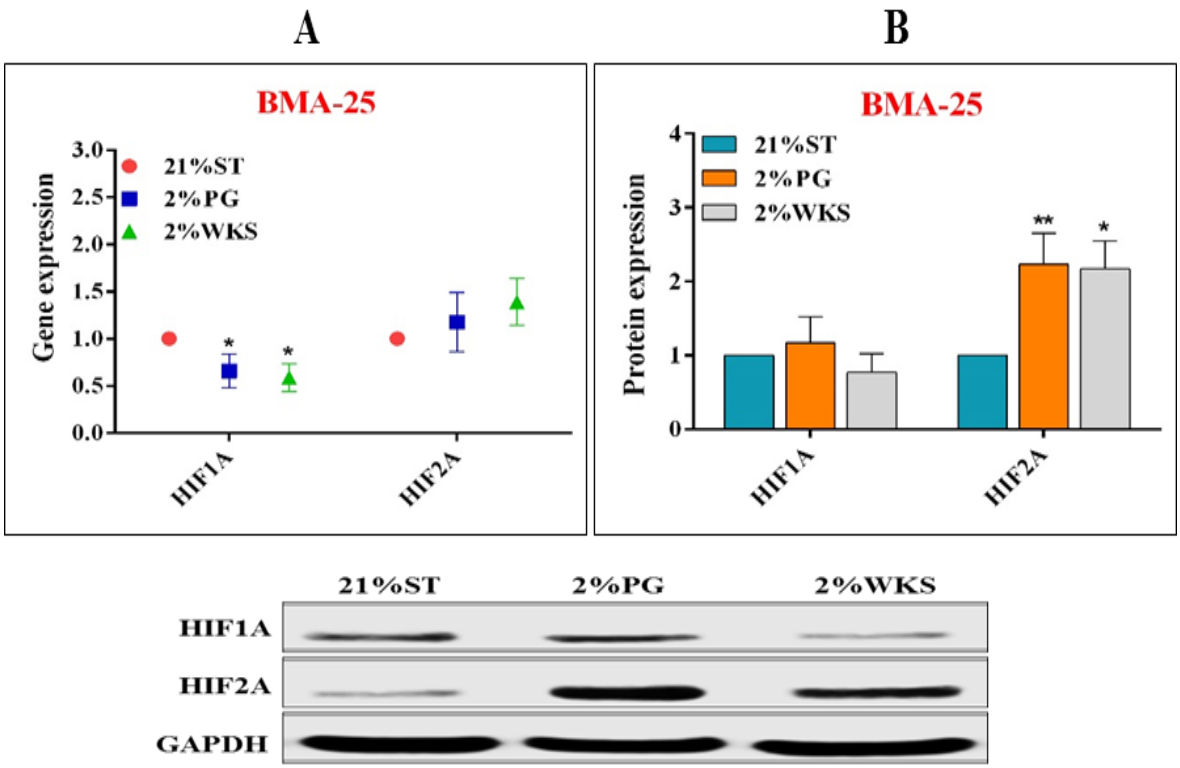


Figure 3.13 HIFs expression in BMA-25 cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

3.4 Discussion

Reduced oxygen is an important environmental factor that influences cells in different contexts. In the last two decades the number of studies exploring how culture conditions affect characteristics and functions of cells have increased considerably. However, MSCs exhibition biological responses to low oxygen in different ways. In addition, reduced oxygen significantly affects major MSCs characteristics such as growth rate, cell viability, differentiation, metabolism and apoptosis (Haque et al., 2013). Furthermore, extensive research in epigenetics marks over the last 10 years has shed light on the role of DNA methylation, revealing a profound impact of these marks on cells development and function (Tsagaratou et al., 2017).

In this chapter, we tested and compared the changes in DNA methylation marks (5mC and 5hmC) and their enzymes (DNMTs and TETs) in human bone marrow-derived mesenchymal stem cells (BM-hMSCs) cultured in three different oxygen conditions. In addition, we measured the characterisation and ability of BM-hMSCs to differentiate as well as their expression of pluripotent markers and HIFs.

Our results demonstrated that the cells possessed the tri-lineage differentiation potential which is characteristic of MSCs **(Figure 3.1)**. Also, along with these positive results of tri-lineage differentiation, BM-hMSCs expressed CD73, CD90 and CD105 positively and lacked expression of CD14, CD19, CD34, CD45 and HLA-DR **(Figure 3.2)**. Interestingly, surface expression of CD73, CD90 and CD105 are more than 95% in both reduced oxygen conditions (2%PG and 2%WKS), whereas the expression of these markers are slightly less than 95% under air oxygen (21%ST). This may be due to prolonged enzymatic time exposure during cell detachment (Brown et al., 2007; Temtem et al., 2009). However, our results showed there was no different between BM-hMSCs cultured in different oxygen conditions.

Our results agree with previous reports that demonstrated that MSCs cultured in either reduced oxygen or air oxygen displayed a typical MSCs profile with regular expression of surface markers overtime and no significant differences observed in cell surface (Holzwarth et al., 2010; Basciano et al., 2011). Moreover, Wang *et al* showed there was no significant differences in MSCs phenotypes before and after treatment with hypoxia (1% O_2) for 24 hours (Wang et al., 2015).

We showed that reduced oxygen did not lead to significant changes in the mRNA levels of OCT-4 and NANOG. In contrast, the expression of SOX-2 was significantly increased in 2% O_2 cultures compared to air oxygen **(Figure 3. 3)**. These results are in line with previous studied that showed the level of SOX-2 significantly increase in hMSCs cultured under low oxygen (5% O_2), but not OCT-4 and NANOG (Ahmed et al., 2016). Similar results also have revealed that the level of pluripotency markers was increased significantly in low oxygen MSCs (D'Ippolito et al., 2006; Fehrer et al., 2007). However, OCT-4 and SOX-2, are naturally expressed in MSCs at low levels in early passages, and their levels gradually decline when the number of passages increases (Liu et al., 2009; Yoon et al., 2011). Therefore, increased levels of OCT-4 and SOX-2 might be involved in maintenance of stemness and in improving MSCs proliferation as well as differentiation capabilities (Han et al., 2014).

RT-qPCR and Western Blot were used to investigate the effect of reduced oxygen tension on the expression of HIF1A and HIF2A subunits. Expression of HIF1A showed a bias toward significant down-regulation by low oxygen, whereas transcription of HIF2A was increased following long-term culture under reduced oxygen conditions **(Section 3.3.7)**. These observations agree with other studies that have shown a significant decrease in mRNA expression of HIF1A after acute or chronic low oxygen incubation (Forristal, et al., 2010; Thienpont et al., 2016). In contrast, western blot and immunocytochemistry showed the

HIF1A protein expressed in reduced oxygen condition is maintained after 10 and 14 days reduced oxygen incubation (Chaven, et al., 2000; Westfall et al., 2008, Forristal, et al., 2010; Lin, et al., 2011). In addition, previous studies have shown that HIF2A was upregulated under hypoxic conditions compared with 21% oxygen following different time point incubation. These results were confirmed by western blot results in different studies (Grayson, et al., 2007; Forristal, et al., 2010; Lin, et al., 2011; Narva, et al.,2013). However, many studies showed that level of HIF1A was increased significantly in MSCs isolated from different sources (Holzwarth et al., 2010; Ranera et al., 2012; Choi et al., 2016). The inconsistency of response of HIFs to low oxygen tension are likely due to the variations in the experimental setup, including (a) the variability between MSCs: in particular source and species (b) the variation between the media composition; (c) subtle changes in the oxygen concentration used (10% to 1%); (d) as well as the capacity of these cells to adapt to reduced oxygen over extended periods of incubation and culture (Müller et al., 2006; Schallmoser et al., 2008; Holzwarth et al., 2010; Ahmed et al., 2016).

Oxygen is an essential factor for several cellular processes in various organisms and, any alternative in its availability, can produce important changes at cellular quantities (Melvin and Rocha, 2012). Moreover, since the discovery that hypoxia is recognised by HIFs enzymes which work as master regulator of cellular adaptation to low oxygen tensions more consideration has been given to the cell culture conditions and how this could alter various epigenetic modifications as well as drive the early developmental stages via regulated the gene expression of cell-type specific (Hitchler and Domann, 2007; Cyr and Domann, 2011; Perez-Perri et al., 2011; Melvin and Rocha, 2012). To date there are a limited number of reports available describing the DNA methylation status in human MSCs, particularly under reduced oxygen tension. However, routine culture of hMSCs in physiological normoxia resulted in a significant decrease in levels of 5mC and 5hmC, accompanied by transcriptional

translational down-regulation of DNMT3B and TET1 in our hands **(Figure 3.6,7 and 3. 8)**. Analysis of methylation status of specific gene promoters by measuring CpGs promoter methylation levels using a pyrosequencing technique indicated that low oxygen alters the DNMT3B promoter methylation levels, but not TET1 **(Figure 3.9 and 3.10)**. Tsai *et al* reported that DNA methylation mediated by DNMT1 is required for the maintenance of BM-MSC properties (Tsai et al., 2012). In addition, altered gene expression during senescence due to specific epigenetic changes is well established (Schellenberg et al., 2011; Schellenberg et al., 2014). Bentivegna *et al* revealed highly significant differences in CpGs methylation levels between early and late passages of BM-hMSCs in almost all chromosomes tested (Bentivegna et al., 2016). In addition, it has been reported that low oxygen MSCs decrease DNA methylation within the promoter regions of OCT-4 and NANOG and this reduction is associated with increased transcriptional levels of the OCT-4, NANOG, SOX-2 and SSEA-4 when compared with air oxygen condition (Tsai et al., 2010). However, while many researchers agree that reduced oxygen is a main factor that improves growth rate, reduce senescence as well as maintains stem cell characteristics of hMSCs (Grayson et al., 2007; Basciano et al., 2011; Tsai et al., 2011), data on the effect of low oxygen culture conditions on DNA methylation status are very limited. However, it has been reported that reduced oxygen caused inhibition of DNMT activity preventing DNA methylation in cancer cells (Shahrzad et al., 2007; Skowronsk et al., 2010). In addition, a recent study indicated that short-exposure (24 hrs) to severe hypoxic (1% O_2), led to changes in the DNA methylation status of primary neuronal cells compared to the control group that were maintained long after the low oxygen tension was removed (Hartley et al., 2013). In contrast, increased DNA methylation has been demonstrated in chronic low oxygen tension studies (Whatson et al., 2009; Melvin and Rocha, 2012). The molecular mechanism behind these changes is still unclear, but several studies have revealed that promoter

hypermethylation prevents HIF binding to the hypoxia response element (HRE) DNA recognition sites, which leads to modulation of the expression of numerous genes that affect cellular survival and metabolism (Skowronski et al., 2010; Deaton and Bird, 2011).

In conclusion, the effects of reduced oxygen *in vitro* cultivation on DNA methylation (5mC and 5hmC) functions are still poorly understood. However, to our knowledge, this is the first work that compares the DNA methylation (5mC and 5hmC) and their enzymes (DNMTs and TETs) of BM-hMSCs that were exposed to different oxygen tension. In this work we have demonstrated that 5mC and 5hmC levels in BM- hMSCs are sensitive to modulation via subtle alterations in the biophysical environment.

Chapter 4: Low oxygen modulates the epigenetic state of pluripotent stem cells and their differentiated progeny cells

4.1 Introduction

Our results in **chapter 3** showed that there were differences in the mean level of DNA methylation (5mC and 5hmC) between physiologic normoxia and air oxygen conditions in BM-MSCs. Also, low oxygen conditions affected BM-MSCs at gene and protein expression. It was therefore considered useful to investigate these effects in human pluripotent stem cells hPS cells. Human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells both share the unique and essential properties of pluripotent stem cells (hPS) cells which is that they can self-renew in culture and differentiate into multiple cell lineages (Tavakoli et al., 2009). These unique abilities give hPS cells a great potential in research, drug screening and therapies for many diseases (Tavakoli et al., 2009; Nii et al., 2014). Moreover, understanding the mechanisms involved in hPS cells differentiation *in vitro* is essential for further understanding of the development of functional cells (Boyer et al., 2005, Galvagni et al., 2015).

Recently, and due to their pluripotency, ES cells have been used as an *in vitro* model system to study epigenetic changes during stem cell differentiation and early development. It has been reported that pluripotency is governed by a complex interaction between genetic and epigenetic basis (Loh, et al., 2008). Evidence illustrates that the epigenetic pattern of undifferentiated human ES cells unique when compared to differentiated and somatic cells (Bibikova et al., 2006; Fouse et al., 2008; Altun et al., 2010).

DNA methylation (5mC) is generated by DNA methyltransferase (DNMT) enzymes by the addition of a methyl group at position C5 of cytosine bases within cytosine-phosphate-guanine dinucleotides (CpG). Functionally, these enzymes have been categorised into two different classes; DNMT1, which is involved in the maintenance of heritable epigenetic marks during cell division, and DNMT3A and DNMT3B, which work in co-ordination with

DNMT1 and are responsible for *de novo* methylation (Okano et al., 1998; Cheng and Blumenthal, 2008; Dhe-Paganon et al., 2011). Recent studies have identified DNMT3L (DNA methyltransferase 3-like) as being highly expressed in human ES cells and interacting with DNMT3A and DNMT3B to stimulate their catalytic activity. However, the precise function of DNMT3L in these cells is not clear (Bourc'his, 2001; Margot et al., 2003). However, a high level of 5-hydroxymethylcytosine (5hmC) was observed in ES cells and some somatic cells suggesting that 5hmC may have a separate role from of 5mC in gene regulation (Tahiliani et al., 2009, Yu et al., 2012). DNA hydroxymethylation is mediated by three TET proteins; TET1, TET2 and TET3 which seem to be differentially expressed in tissues; TET1 being mainly expressed in ES cells and TET2 and TET3 expressed more universally (Tsagaratou et al., 2017). However, the biological role of 5hmC and the associated enzymes in cells has not yet been completely elucidated (Tan and Shi, 2012; Tellez-Plaza et al, 2014).

Human ES cells are routinely cultured under air room oxygen tensions (21% O_2) but are derived from embryos which reside in a 2–5% physiological normoxia environment (Forristal et al., 2010). However, the stem cell niche is involved in the regulation of stem cell behaviour through balanced mechanisms controlling production of stem cell and progenitor cells which act to maintain homeostasis (Voog and Jones, 2010; Papagiannouli and Lohmann, 2015). It has established that reduced oxygen tensions are beneficial for the maintenance of human ES cell pluripotency, reduced spontaneous differentiation, and improved genetic stability (Forsyth et al., 2006; Ludwig et al., 2006). Cell response to low oxygen tension is mediated by members of the hypoxia-inducible factors (HIFs). Many studies have revealed that both HIF1A and HIF2A are critical for self-renew and maintenance of the undifferentiated state of human ES cells (Gordan and Simon, 2007). In addition, evidence has suggested that epigenetic regulation controls the adaptation of stem

cells to changes in the physiological environment (hypoxia) and play a crucial role in maintenance of homeostasis (Waston et al., 2010; Robinsons et al., 2012). Recently, it was reported that DNA methylation-controlled HIFs activation through regulation of transcription of hypoxia response elements (HRE) and controlling the HIFs stabilization (Waston et al., 2014).

Understanding the functional significance of genes involved in prevention of DNA methylation modification against changes in the physiological environment may help provide a better understanding of the major underlying mechanisms that cover the pluripotency of stem cells and cellular diversity, leading to improved efficacy and safety in the use of stem cells for applied and basic purpose in research, drug screening and therapy. The quality and status of cells are important before continuing with any further studies, the aim of this chapter was to determine the functional characterisation of hPS cells and their differentiation progeny and to study the role of two reduced oxygen conditions (2%PG and 2%WKS) in regulation of epigenetic marks in human pluripotent stem cells (hPSCs).

4.2 Aims

1-Investigate the effects of reduced oxygen on characteristics of hES cells compared with air oxygen.

2. Investigate the effects of physiological normoxia on global DNA methylation (5mC and 5hmC) and examine local patterns within the promoter regions of specific genes in hPS cells and their differentiated progeny in comparison to air oxygen tensions.

3. Assess the relationship between changes in global DNA methylation (5mC and 5hmC) patterns in response to oxygen modulation and the transcriptional expression of DNMTs/TETs.

4.3 Methods

4.3.1 Human PSCs culture

Human PSCs were cultured, maintained and expanded in xeno-free E8M under three oxygen conditions as described in **Section 2.2.1** and **Section 2.2.3**. To maintain the oxygen level of the cells during physiological oxygen culture, cell culture media was deoxygenated to predefined oxygen concentration (2% O_2) as described in **Section 2.2.2**.

4.3.2 Spontaneous differentiation

Spontaneous differentiation was performed as described in **Section 2.3.2,** and the evaluation of pluripotent markers expression in human pluripotent stem cells and their progeny was undertaken as shown in **Section 2.3.4**.

4.3.3 Human pluripotent stem cells characterisation

Undifferentiated hPS cells was seeded at a density of 3.5 x10^4 cells/cm^2 in a 6 well plate and maintained in Essential 8 basal Medium over 96 hours prior to immunocytochemistry as described in **Section 2.3.3.1**. Flow cytometry of undifferentiated hPS cells was performed as described in **Section 2.3.3.2**.

4.3.4 Measurement of cellular viability

Changes in cellular viability following hypoxia treatment were assessed as outlined in **Chapter 2, Section 2.6**.

4.3.5 Global 5mC and 5hmC analysis

Total genomic DNA was isolated from the hPS cells and their differentiated progeny at different time points as described in **Section 2.7.2**. DNA, 100ng and 200ng, was subjected to methylated DNA (5mC) and hydroxymethylated DNA (5hmC) quantification assay

(Colorimetric), respectively, with the MethylFlash quantification kit as outlined in **Section 2.7.3**.

4.3.6 Quantitative real-time RT-PCR

Total RNA was extracted from the hPS cells and their differentiated progeny at different time points as described in **Section 2.8.1**. Relative gene expression was carried out in triplicate samples by using the QuantiFast SYBR Green OneStep RT-PCR kit **(Section 2.8.3)**.

4.3.7 Protein analysis

Undifferentiated hPS cells and their differentiation cells were cultured on 6 well-plate and cells lysed as described in **Section 2.9.1** before 30µg of total protein **(Section 2.9.2)** was subjected to western blot analysis using antibodies against DNMT3B, TET1, HIF1A and HIF2A as described in **Chapter 2, Section 2.9.4**.

4.3.8 Pyrosequencing of sodium bisulphite-converted DNA

500ng of genomic DNA was subjected to bisulphite conversion before pyrosequencing was achieved using a PyroMark Gold Q24 Reagents and PyroMark Q24 Software 2.0 **(Full details in Chapter 2, Section 2.10)**.

4.4 Results

4.4.1 Effect of reduced oxygen on the proliferation of hPS cells

A cell counting assay was used to determine proliferation of the hPS cells (ESCSs and hiPSCs) cultured in air oxygen (21%ST) and reduced oxygen conditions (2%PG and 2%WKS). The number of cells seeded into multiple wells of a 6-well plate were counted daily using a counting chamber for 6 days. As shown in (**Figure 4.1A, B, C)**, in both

conditions culture of physiological normoxia (2%PG and 2%WKS), there was a significant increase in cell proliferation between days 2 and 6 in comparison to those cultured in air oxygen (21%ST, $p<0.05$ or $p<0.01$). hPS cells cultured under 2%WKS condition had a higher proliferation rate ($p<0.01$) than those in 21%ST and this statistically significant increase in the cell count remained consistent in hPS cells cultured in 2%WKS conditions till day 6 of the experiment. The results also showed there was no difference in the proliferation rate between two reduced oxygen conditions **(Figure 4.1A, B, C)**. The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was increase in the proliferation rate of cells cultured under reduced oxygen conditions (2%PG and 2%WKS), but no significant differences were observed when compared to air oxygen conditions - probably because of the variability between three cell lines according to cell line-specific biases **(Figure 4.1D)**.

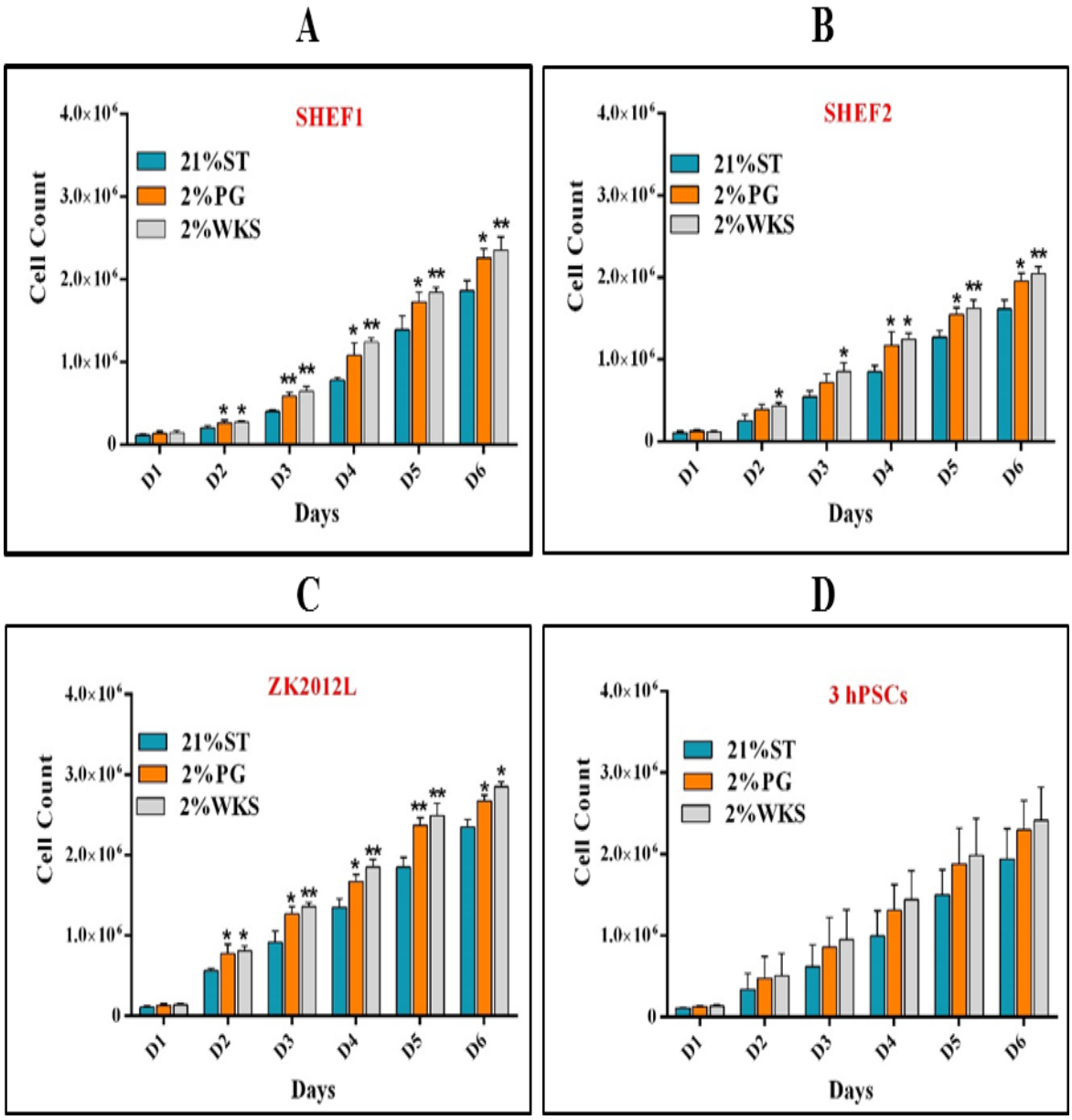


Figure 4.1 Effect of reduced oxygen on the proliferation rate of hPS cells. A) SHEF1 B) SHEF2 C) hiPSCs line (ZK2012L) D) 3 hPSC line (SHEF1, SHEF2 and ZK2012L). Cell count experiments were conducted over 6 days after incubation in air oxygen (21%ST) and physiological normoxia conditions (2%PG and 2%WKS), with media changed every day. Y-axis indicates cell number x 10^6. X-axis indicates time (days). Data are shown as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.4.2 Reduced oxygen increases metabolic activity in hPS cells

MTT and Alamar blue assays were used to analyse the metabolic activity of hPS cells cultured in air oxygen (21%ST) and reduced oxygen conditions (2%PG and 2%WKS). Similar to above an increase in the number of viable cells quantified by MTT assay was noticed for hPS cells cultured in reduced oxygen conditions **(Figure 4.2)**. SHEF1 displayed a significant increase in MTT at days 5 and 6 in both reduced oxygen conditions ($p<0.01$) **(Figure 4.2A).** ZK2012L cells showed significantly higher proliferation potential in in 2%WKS than those cultured in air oxygen and 2%PG ($p<0.01$) whereas SHEF2 exhibited significant MTT increases in both reduced oxygen conditions ($p<0.05$) at day 5 and 6. **(Figure 4.2B, C)**. The results also showed that cell viability of ZK2012L cells was increased significantly in 2%WKS as compared with 2%PG at day 5. The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was increased in the metabolic activity of cells cultured under reduced oxygen conditions (2%PG and 2%WKS), but there were no significant differences was observed when compared to air oxygen conditions **(Figure 4.2D)**.

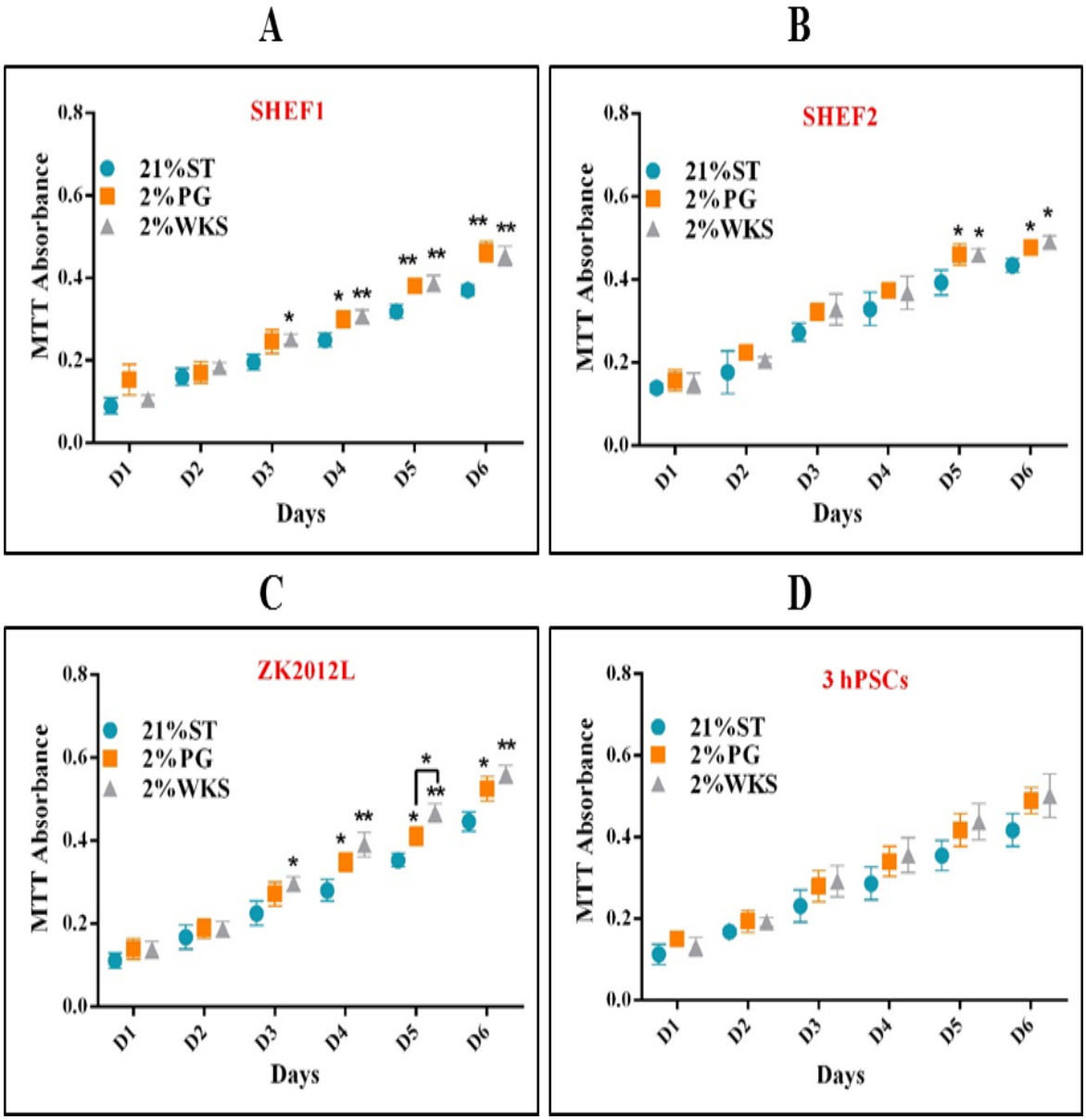


Figure 4.2 Effect of reduced oxygen on the viability of hPS cells. A) SHEF1 B) SHEF2 C) hiPSCs line (ZK2012L) D) 3 hPSC line (SHEF1, SHEF2 and ZK2012L). MTT absorbance (570 nm) was measured over 6 consecutive days in hPS cells incubated under air oxygen (21%ST) and physiological normoxia conditions (2%PG and 2%WKS). Y-axis indicates MTT absorbance (570 nm). X-axis indicates time (days). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Likewise, SHEF1 displayed a high significant increase in Alamar blue absorbance at day 5 and 6 in both hypoxia conditions ($p<0.01$) **(Figure 4.3A)**. SHEF2 exhibited significant

increases in both physiological normoxia conditions ($p<0.05$) at day 4, and thereafter (**Figure 4.3B)**. ZK2012L cells displayed a high significant increase at day 5 and 6 ($p<0.01$) in both hypoxic cultured **(Figure 4.3C)**. The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was a significant increase in the Alamar blue absorbance of cells cultured under both reduced oxygen conditions between days 4 and 6 in comparison to those cultured in air oxygen (21%ST, $p<0.01$) **(Figure 4.3D)**.

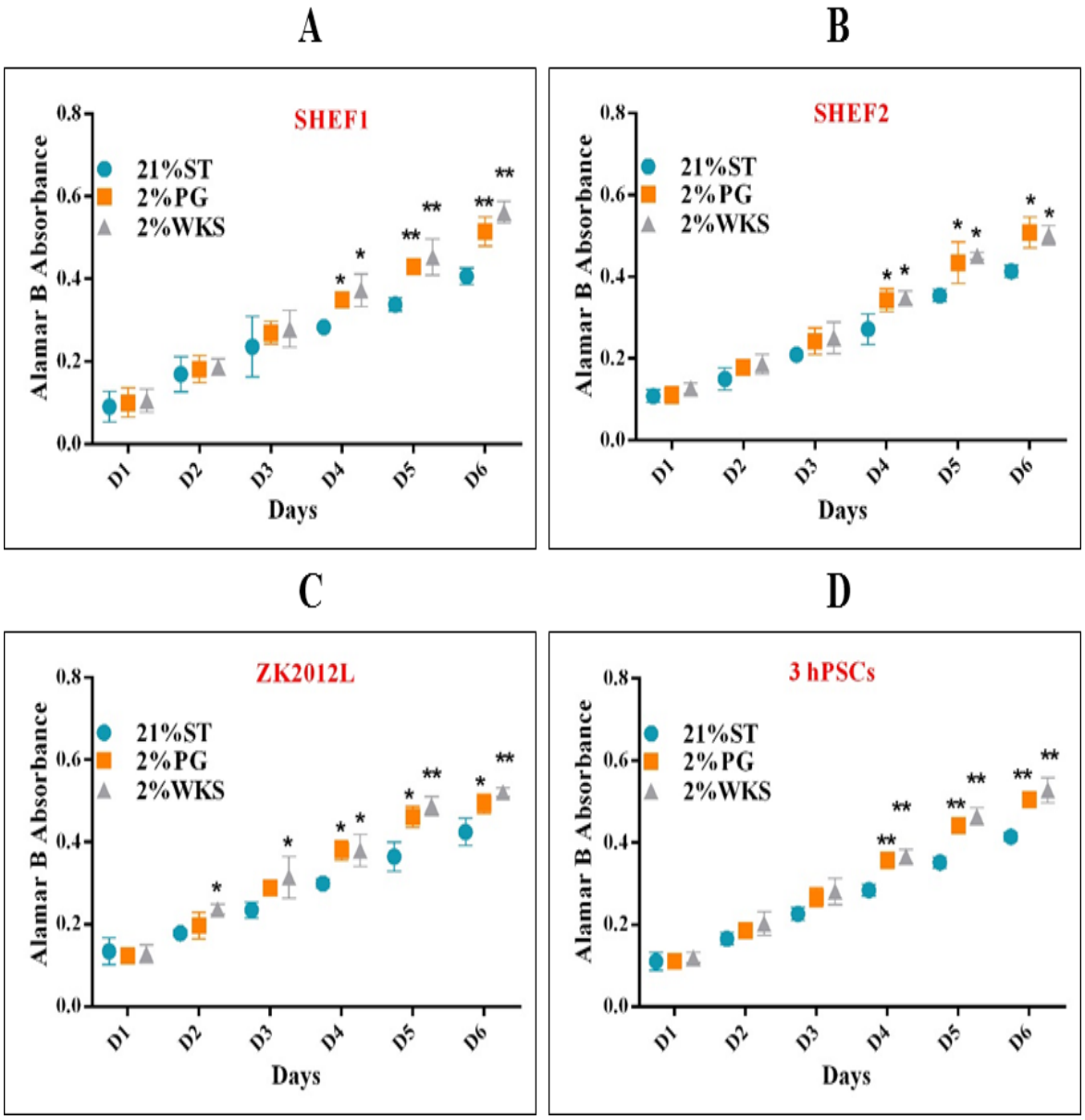
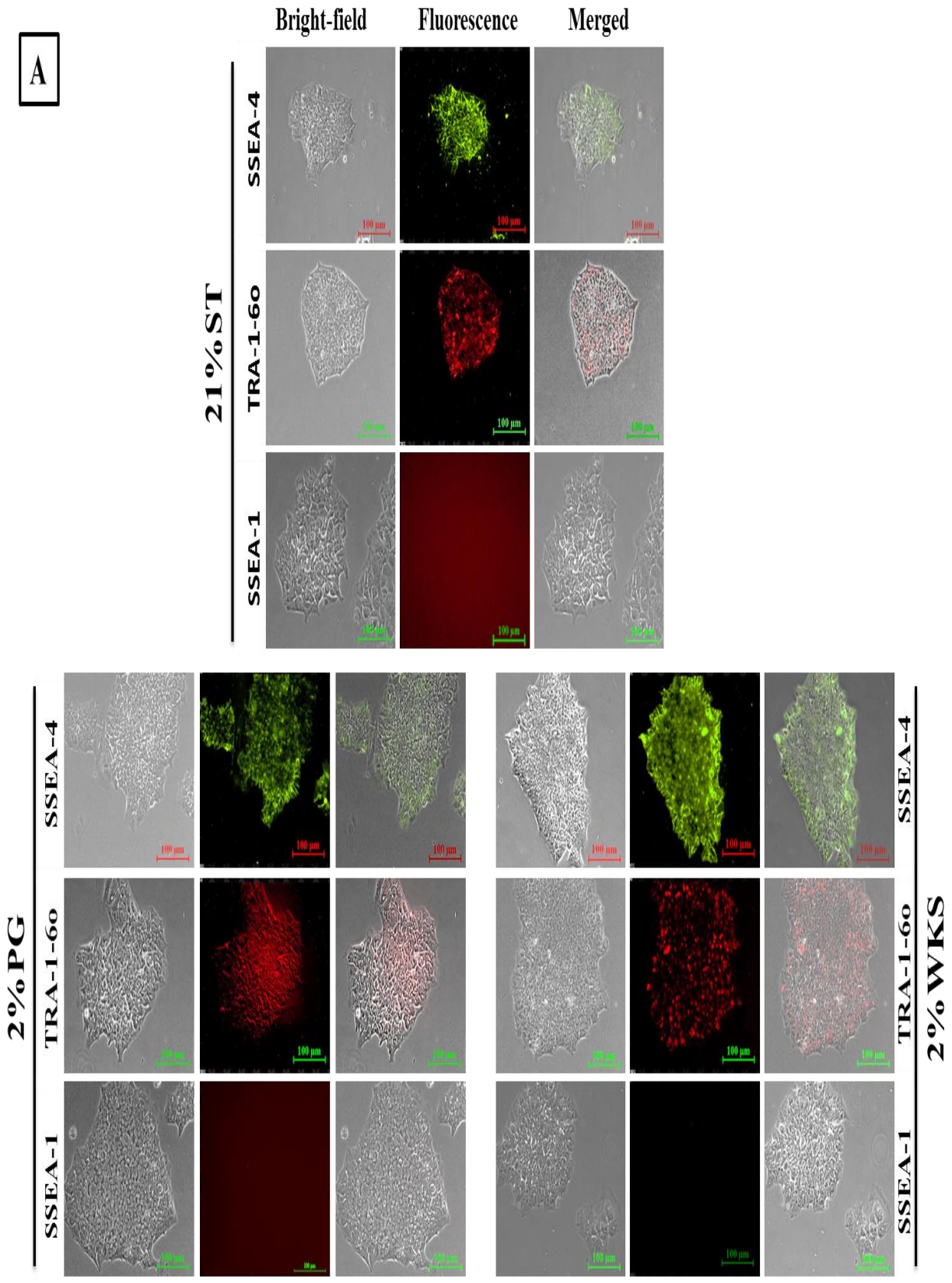


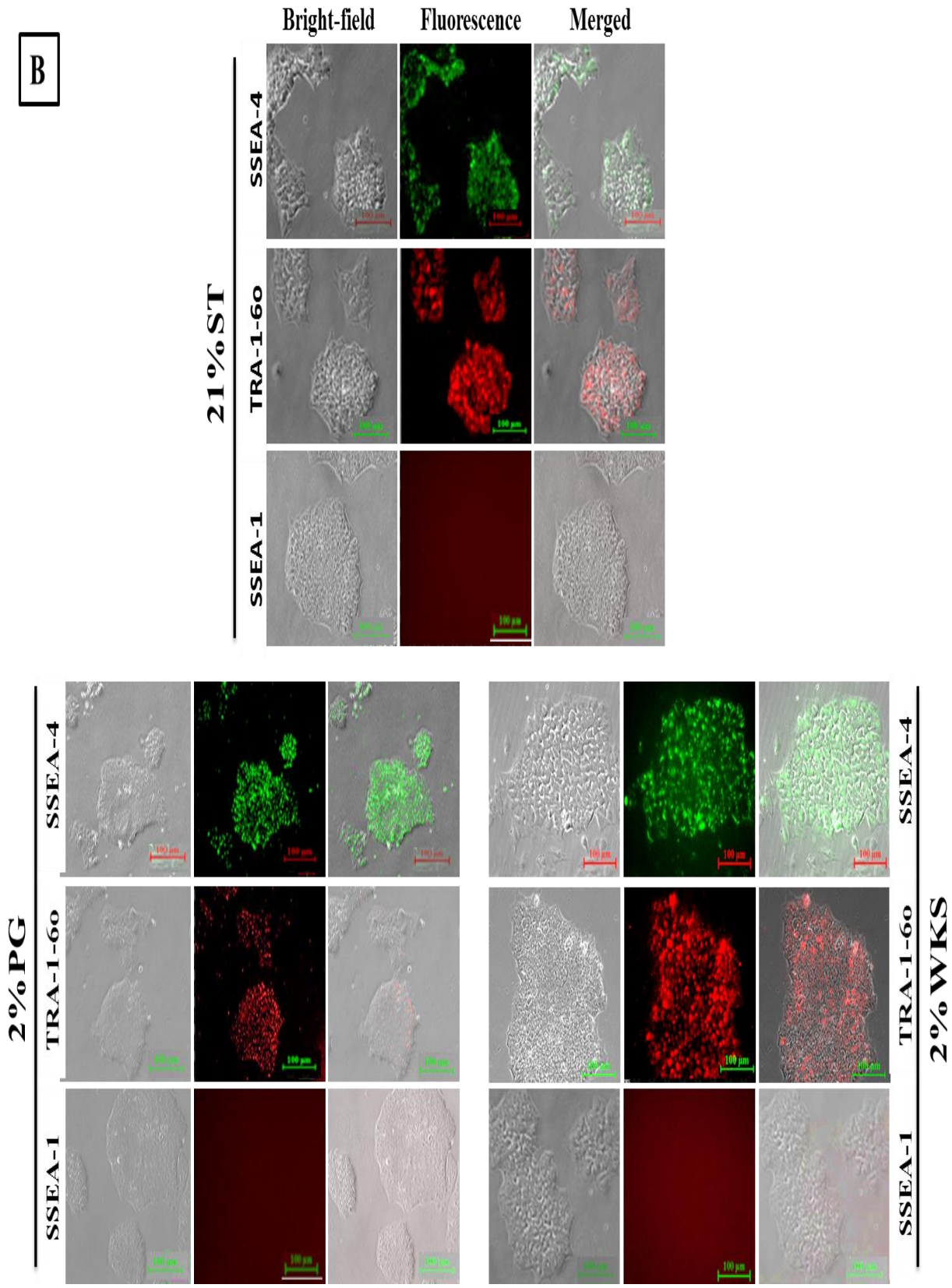
Figure 4.3 Changes in cell viability of hPS cells cultured under reduced oxygen conditions. A) SHEF1 B) SHEF2 C) hiPSCs line (ZK2012L) D) 3 hPSC line (SHEF1, SHEF2 and ZK2012L). Alamar blue absorbance (570 nm) was conducted over 6 days after

incubation in air oxygen (21%ST) and physiological normoxia conditions (2%PG and 2%WKS). Y-axis indicates Alamar blue absorbance (570 nm). X-axis indicates time (days). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.4.3 Characterisation of hPS cells following treatment with low oxygen tension

The expression of pluripotency markers SSEA-4, TRA- 1-60 and differentiation markers SSEA-1 were then explored via a combination of immunocytochemistry and flow cytometry in undifferentiated hPS cells. As shown in **Figure 4.4**, live, unfixed cells displayed positive expression of the pluripotency surface markers SSEA-4 and TRA-1-60 and no expression of the SSEA-1 marker. This demonstrates that a typical hPS cells surface marker expression is retained in all oxygen conditions.


A
Bright-field
Fluorescence
Merged
21%ST
SSEA-4
TRA-1-60
SSEA-1
2%PG
2%WKS
100 µm


B
Bright-field
Fluorescence
Merged
21%ST
SSEA-4
TRA-1-60
SSEA-1
2%PG
2%WKS

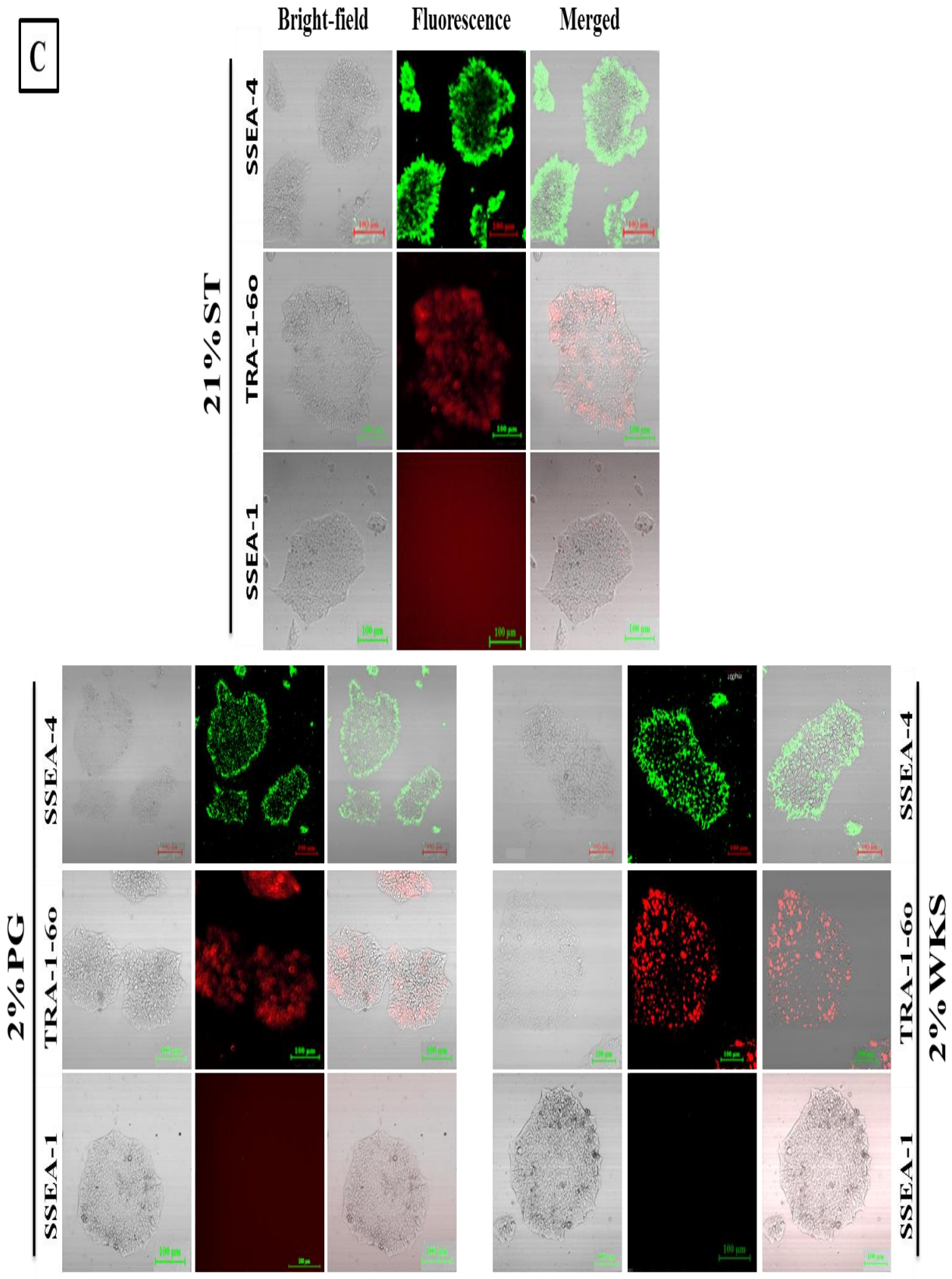
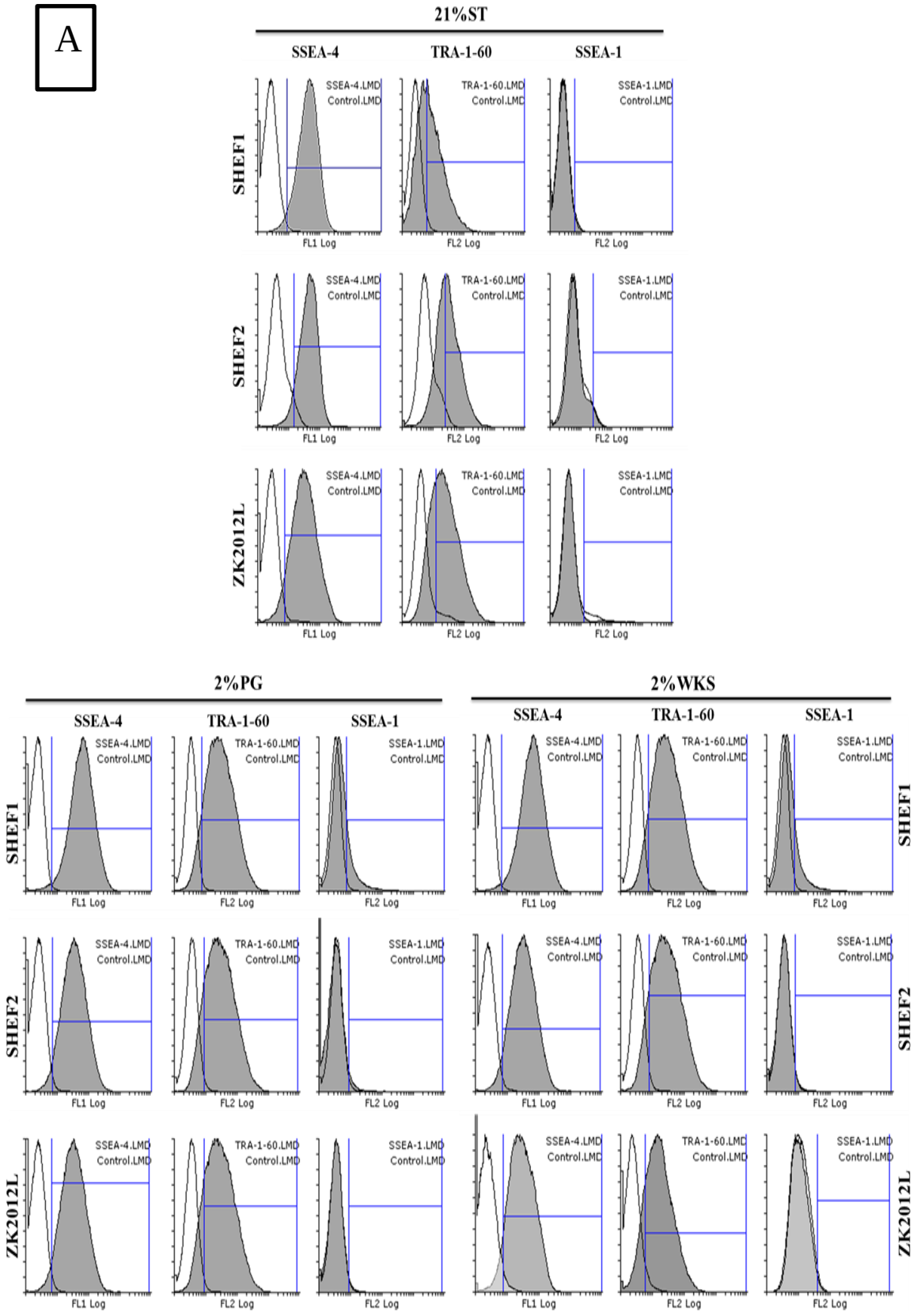


Figure 4.4 Immunocytochemical staining of live, unfixed hPS cells. A) SHEF1 B) SHEF2 C) hiPSC line (ZK2012L). Cells were seeded at a density of 35,000 cells/cm^2 in 6-well plates in three different oxygen tensions and left for 96 hours. The left column shows phase-contrast images, middle column shows fluorescent images and right column shows merged

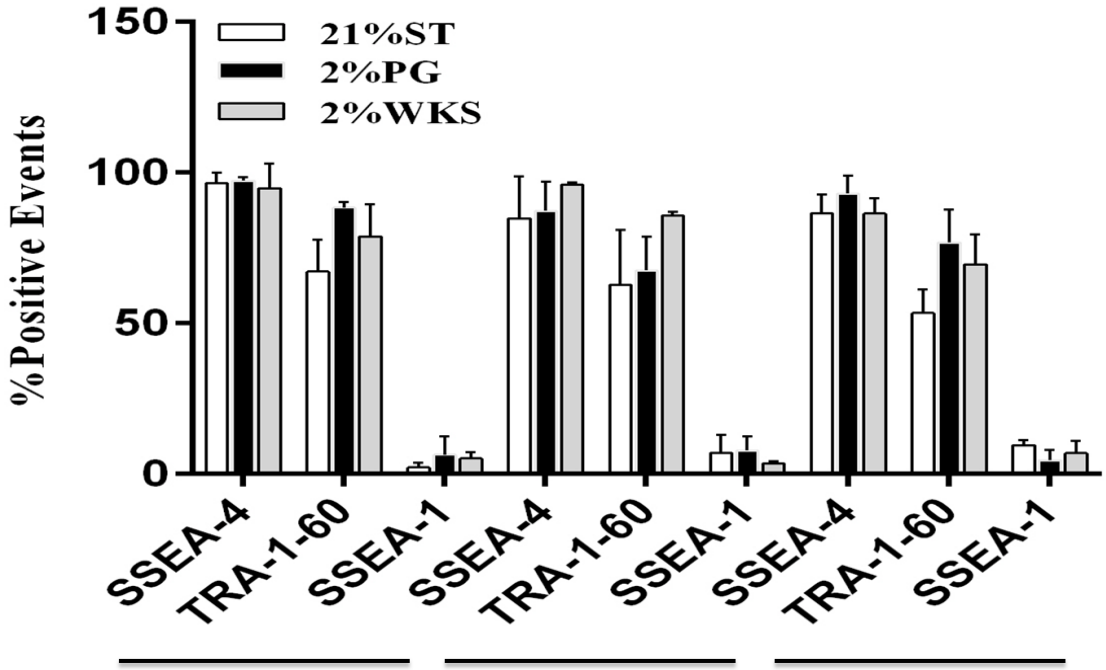
images. bright-field for 4X objective and phase contrast for 10X and 20X objectives. The scale bar represents 100 µm.

To further characterize pluripotency status, the expression of cell surface markers was quantified by flow cytometry **(Figure 4.5)**. The results as a percentage of positive events showed that SHEF1 displayed 96.61 ± 3.41, 94.78 ± 8.31 and 97.36 ± 1.09 for SSEA-4; 67.23 ± 10.56, 78.76 ± 10.68 and 88.41 ± 1.78 for TRA-1-60; and 2.23 ± 1.41, 5.15 ± 2.18 and 6.44 ± 5.94 for SSEA-1, in three oxygen tension conditions (21%ST, 2%PG and 2%WKS), respectively **(Figure 4.5).** SHEF2 displayed 84.76 ± 14.09, 87.29 ± 9.75 and 96.02 ± 0.66 for SSEA-4; 62.71 ± 18.17, 67.53 ± 11.22 and 85.64 ± 1.26 for TRA-1-60; and 7.12 ± 5.9, 7.84 ± 4.57 and 3.39 ± 0.77 for SSEA-1, in three oxygen tension conditions (21%ST, 2%PG and 2%WKS), respectively. hiPSC line (ZK2012L) displayed 86.54 ± 6.3, 93.04 ± 6.02 and 86.4 ± 5.22 for SSEA-4; 53.48 ± 7.66, 76.84 ± 0.84 and 69.61 ± 9.8 for TRA-1-60; 9.5 ± 1.84, 4.53 ± 3.53 and 6.9 ± 3.92 for SSEA-1, in three oxygen tension conditions (21%ST, 2%PG and 2%WKS), respectively. These results demonstrated that three cell lines expressed the hPS cells markers at equivalent levels for the three oxygen tensions in which the cells were incubated.

A


21%ST
SSEA-4
TRA-1-60
SSEA-1
SHEF1
SHEF2
ZK2012L
SSEA-4.LMD
Control.LMD
TRA-1-60.LMD
SSEA-1.LMD
FL1 Log
FL2 Log
2%PG
2%WKS

B



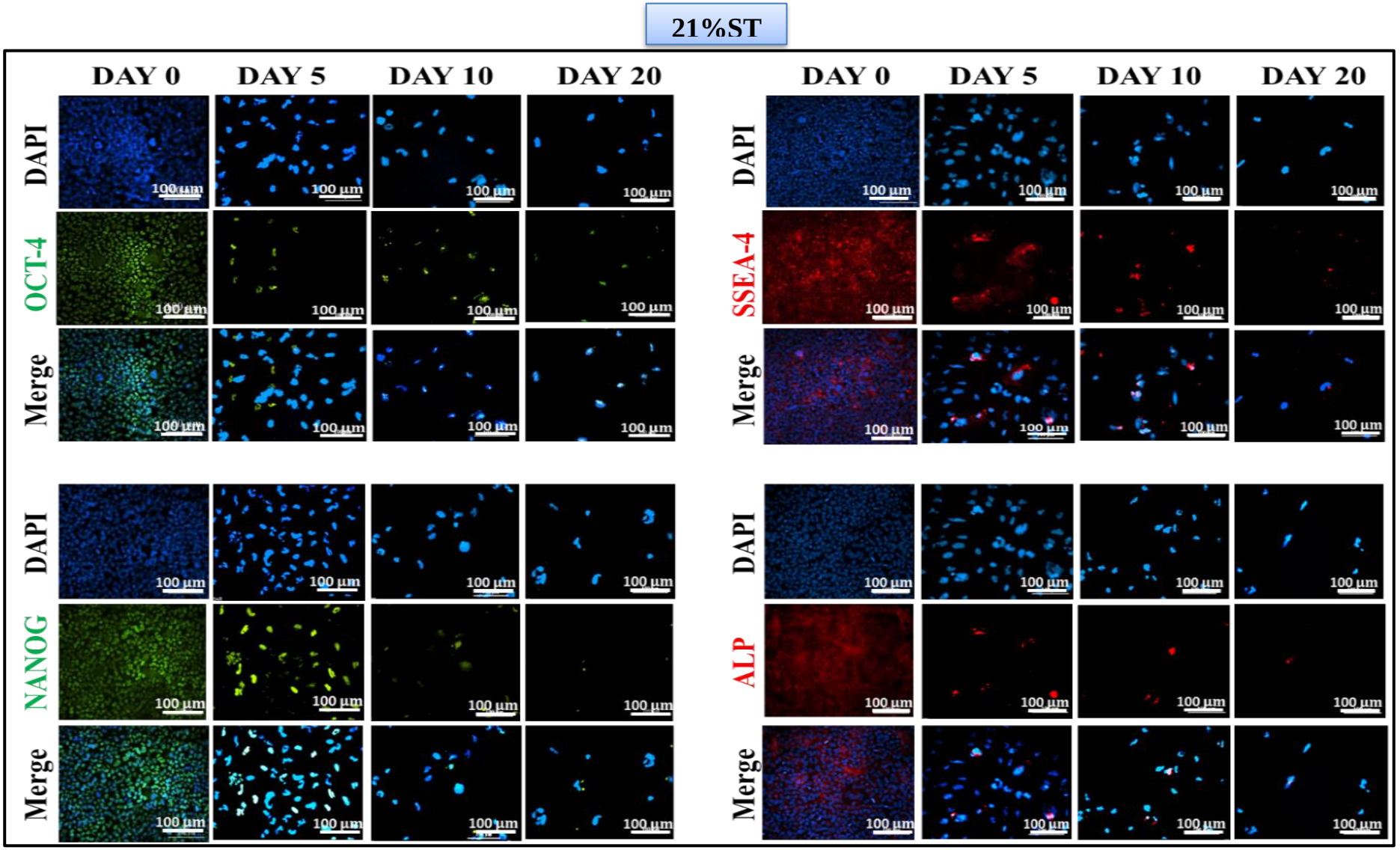
	SHEF1			SHEF2			ZK2012L		
21%ST	96.61	62.71	2.23	84.76	62.71	7.12	86.55	53.48	9.5
2%PG	97.36	88.41	6.45	87.3	67.53	7.84	93.04	76.84	4.53
2%WKS	94.78	78.76	5.2	96.03	85.64	3.38	86.4	69.61	6.9
	SSEA-4	TRA-1-60	SSEA-1	SSEA-4	TRA-1-60	SSEA-1	SSEA-4	TRA-1-60	SSEA-1

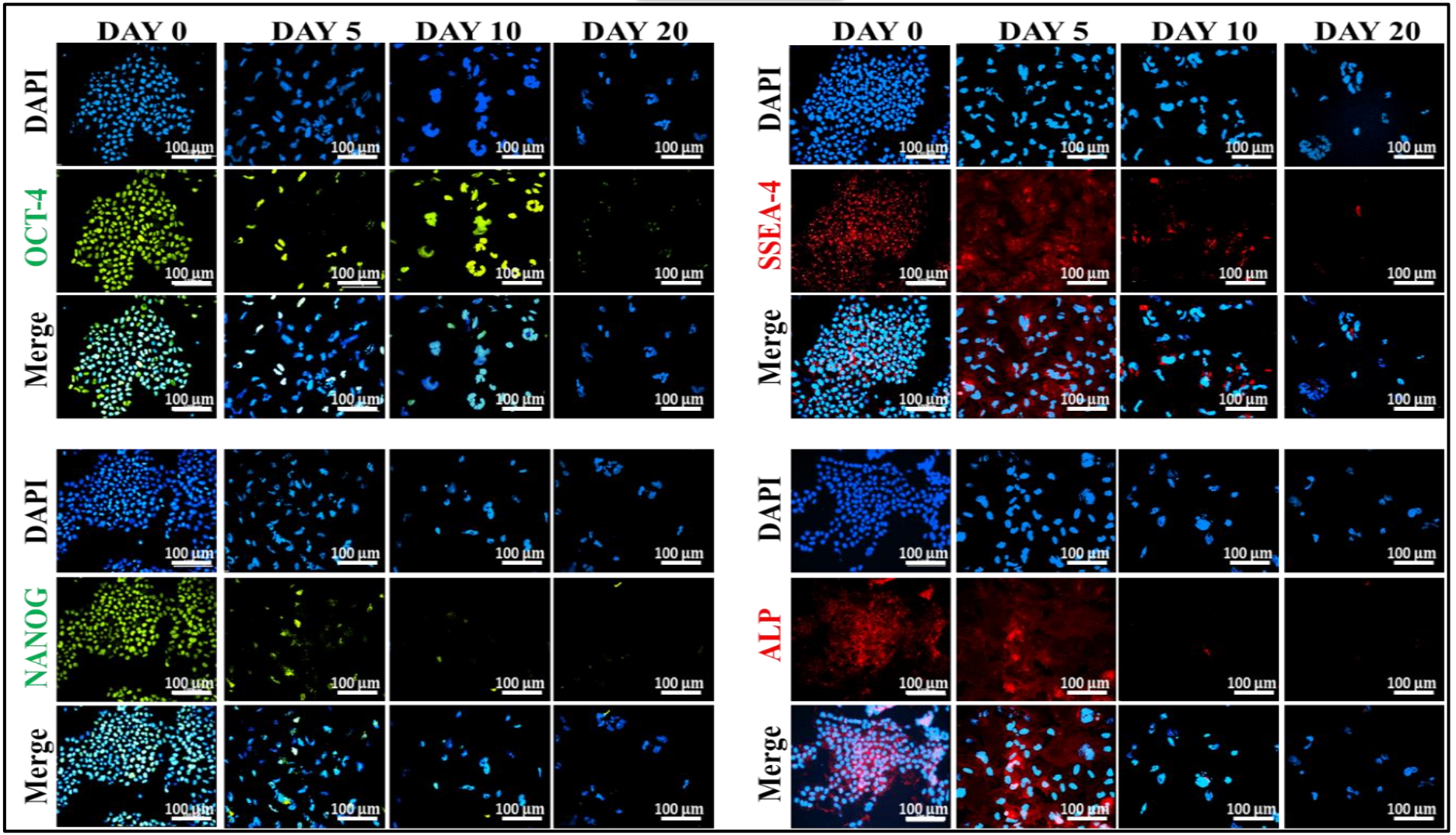
Figure 4.5 Pluripotent marker expression of hPS cells. Pluripotent marker expression of hPS cells cultured under three oxygen tensions were tested by FACS analysis. A) Overlay histograms of hPS cells by Flowing Software. B) All cell samples were found to be positive for both SSEA-4 and TRA-1-60, and negative for SSEA-1 after each experiment at both reduced oxygen (2%PG and 2%WKS) and air oxygen (21%ST). Y-axis indicates surface markers. X-axis indicates % of positive events. Data are presented as mean ± standard deviation (SD). n=3.

4.4.4 Evaluation of spontaneous differentiation of hPS cells

Expression of OCT-4 and SSEA-4 along with NANOG and ALP were analysed by immunofluorescence in undifferentiated cells (day 0) and from spontaneous differentiation (5, 10 and 20 days) in hPS cells. The results showed that all markers (OCT-4, SSEA-4,

NANOG and ALP) were expressed in high levels in undifferentiated hPS cells under all conditions tested (21%ST, 2%PG and 2%WKS). The results also showed that the expression of pluripotent markers decreased from 0 to 20 days in a time-dependent manner. SHEF1 in 21%ST showed a reduction in OCT-4, SSEA-4, and ALP expression after 5 days, and NANOG after 10 days of differentiation. Also, 2%PG showed a notable reduction in OCT-4 and NANOG after 5 and 20 days of differentiation, respectively, while SSEA-4, and ALP showed reduction at day 10 of differentiation. OCT-4 in 2%WKS condition was reduced after day 5 of differentiation, while expression of NANOG, SSEA-4, and ALP showed notable reduction in day 10 of differentiation **(Figure 4.6)**. OCT-4, NANOG and ALP in SHEF2 showed remarkable reduction expression in day 10 of differentiation in 21%ST, 2%PG, and 2%WKS, respectively, while expression of SSEA-4 showed notable reduction after 10 days in 21%ST, and in day 20 of differentiation in %PG and 2%WKS, respectively **(Figure 4.7)**. hiPSC line (ZK2012L) showed remarkable reduction in OCT-4 and SSEA-4 expression in day 10 of differentiation in 21%ST, 2%PG, and 2%WKS, respectively, and NANOG after 10 days in 21%ST and 2%PG, and in day 5 of differentiation in 2%WKS. ALP showed notable reduction in day 5 of differentiation in 21%ST, and after 10 of differentiation in 2%PG, and 2%WKS, respectively **(Figure 4.8)**. In conclusion, undifferentiated hPS cells showed positive expression of OCT-4, SSEA-4, NANOG and ALP while the expression of these markers was reduced during cell differentiation with an increase in the size of the cells.


21%ST
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm


2%PG
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm

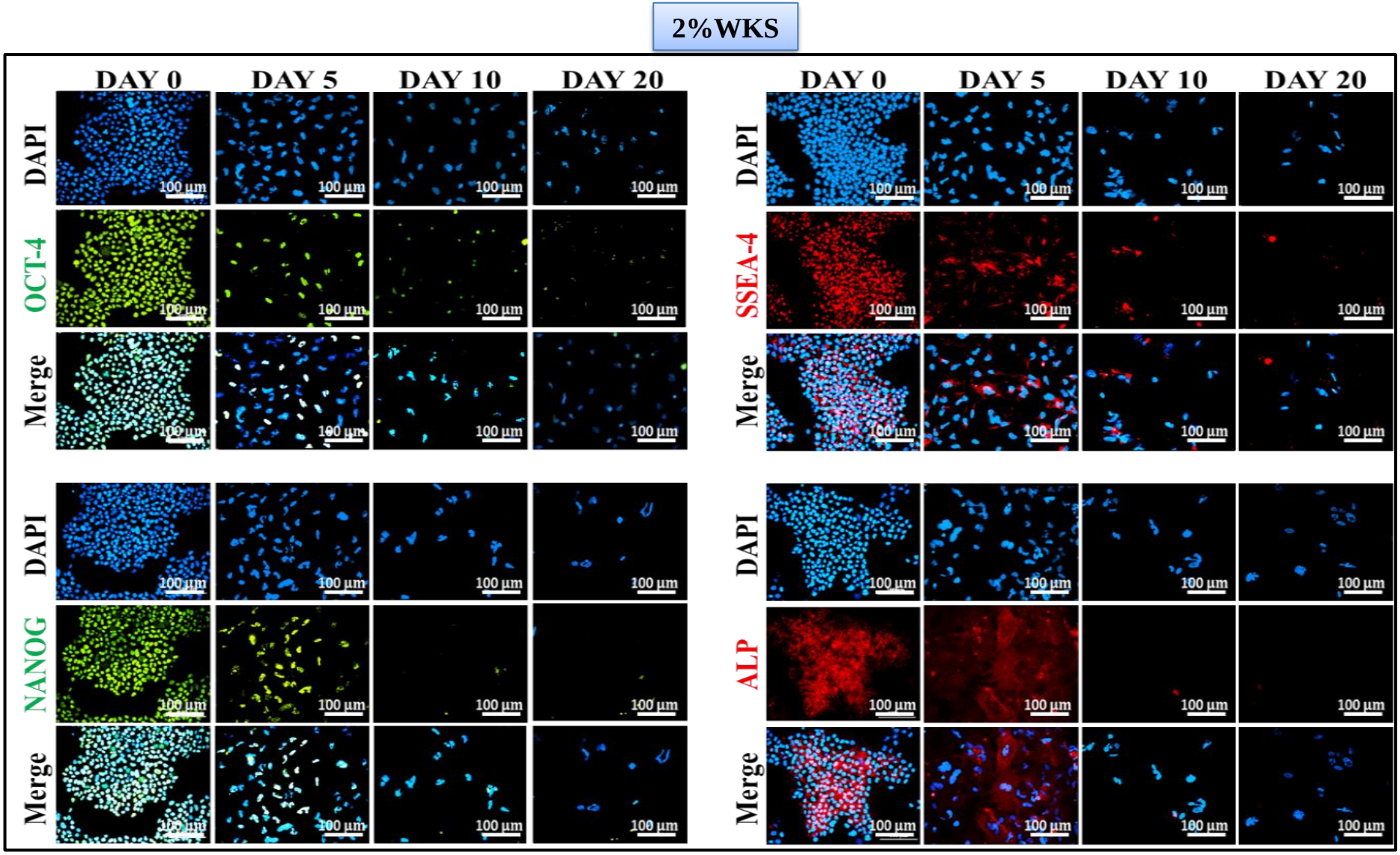
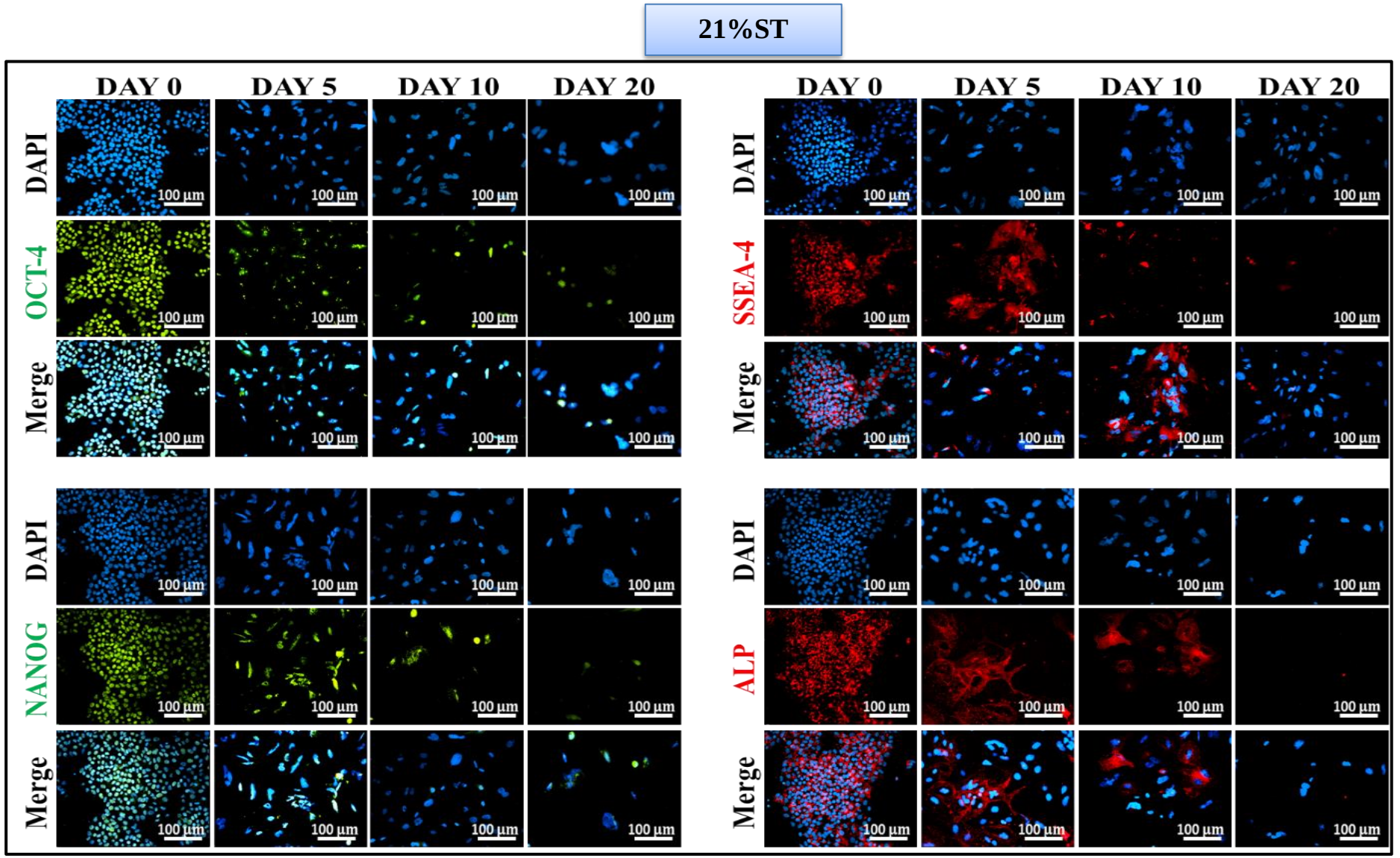
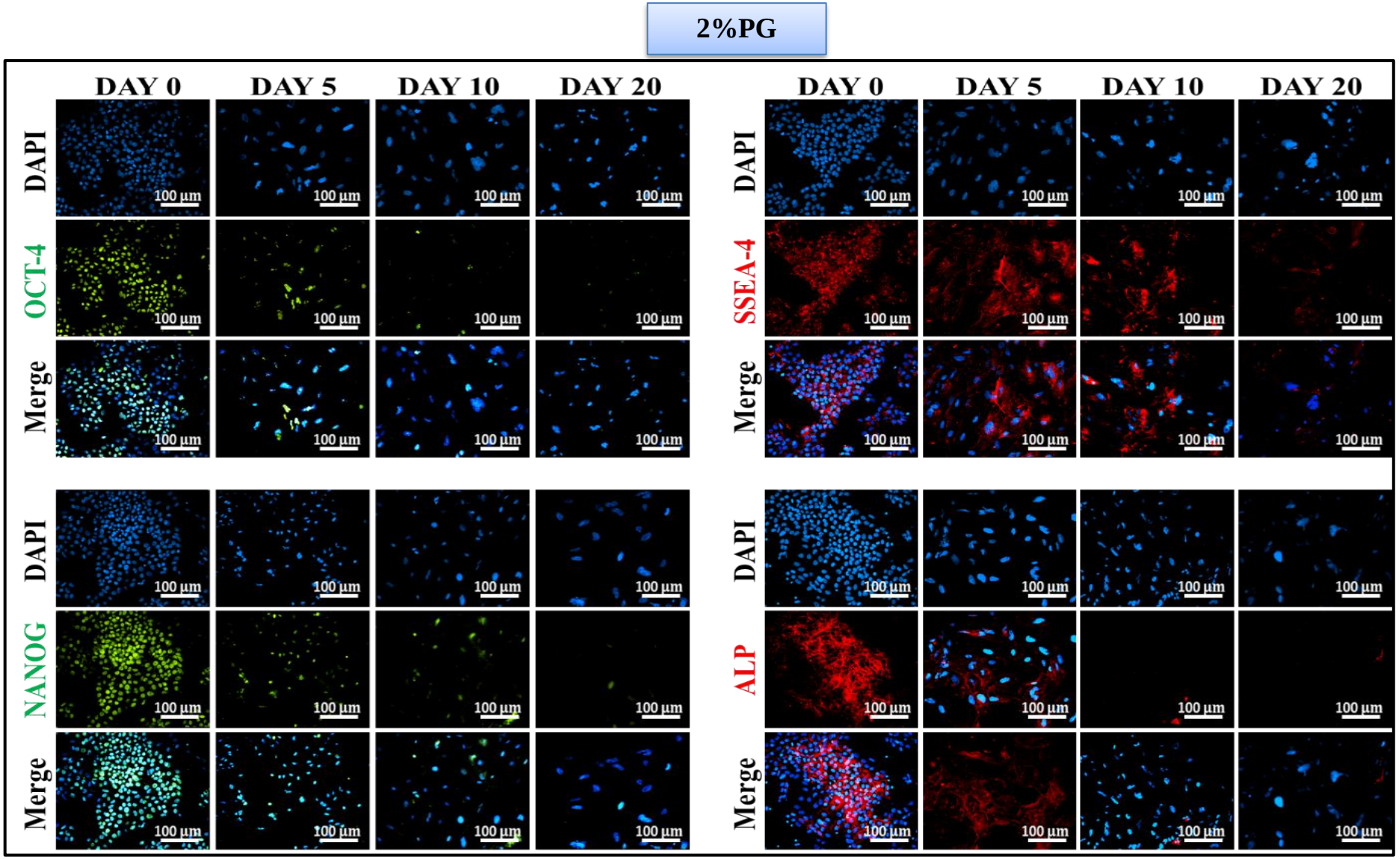


Figure 4.6 Immunofluorescence staining for pluripotency markers in undifferentiated and differentiating SHEF1 cells. Cells were seeded in 24 well-plates in three different oxygen tensions at selected time points (0, 5, 10 and 20 days). Red and green colour indicates antigen, blue DAPI. Each scale bar represents 100 µm.


21%ST
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm


2%PG
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm

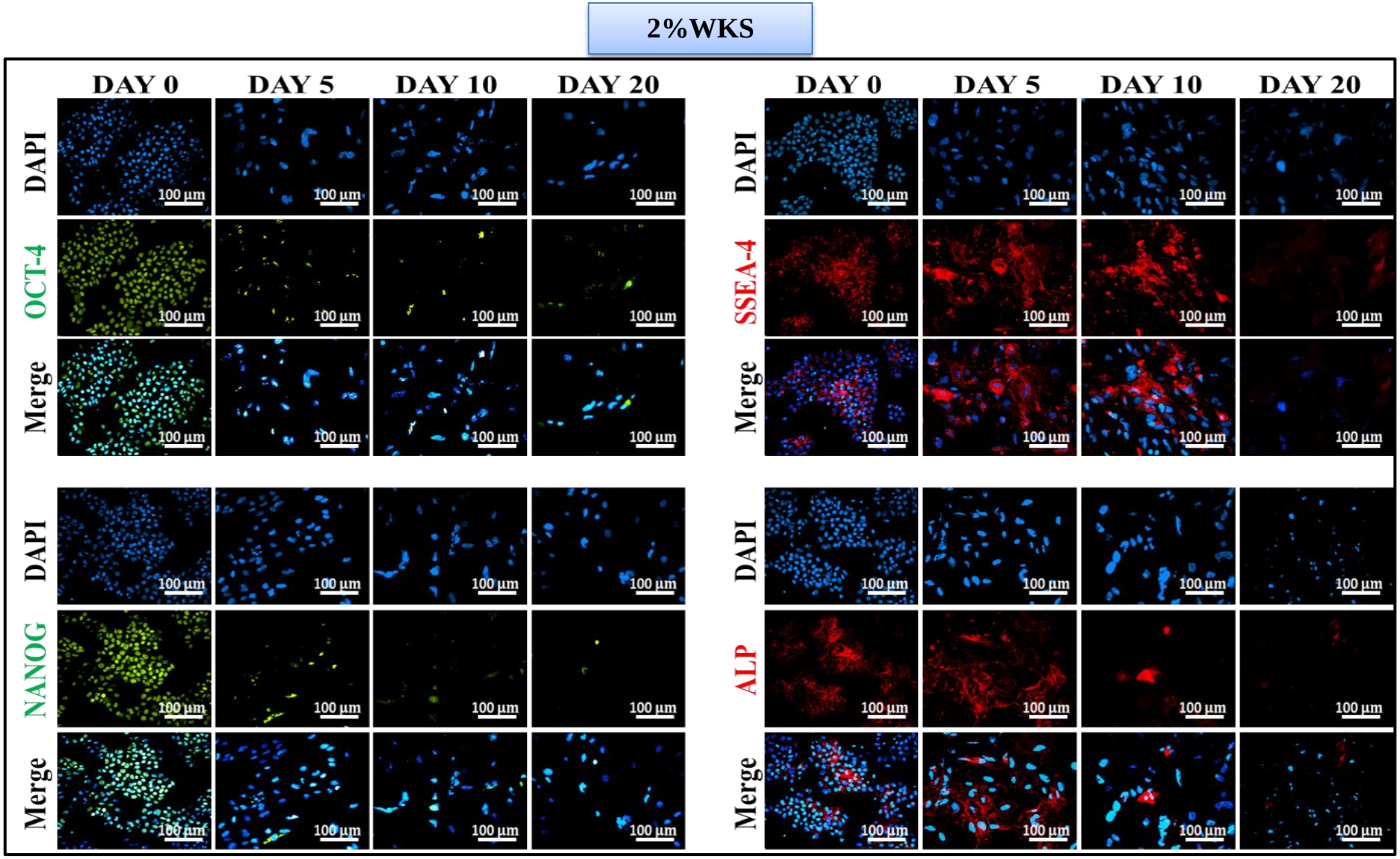
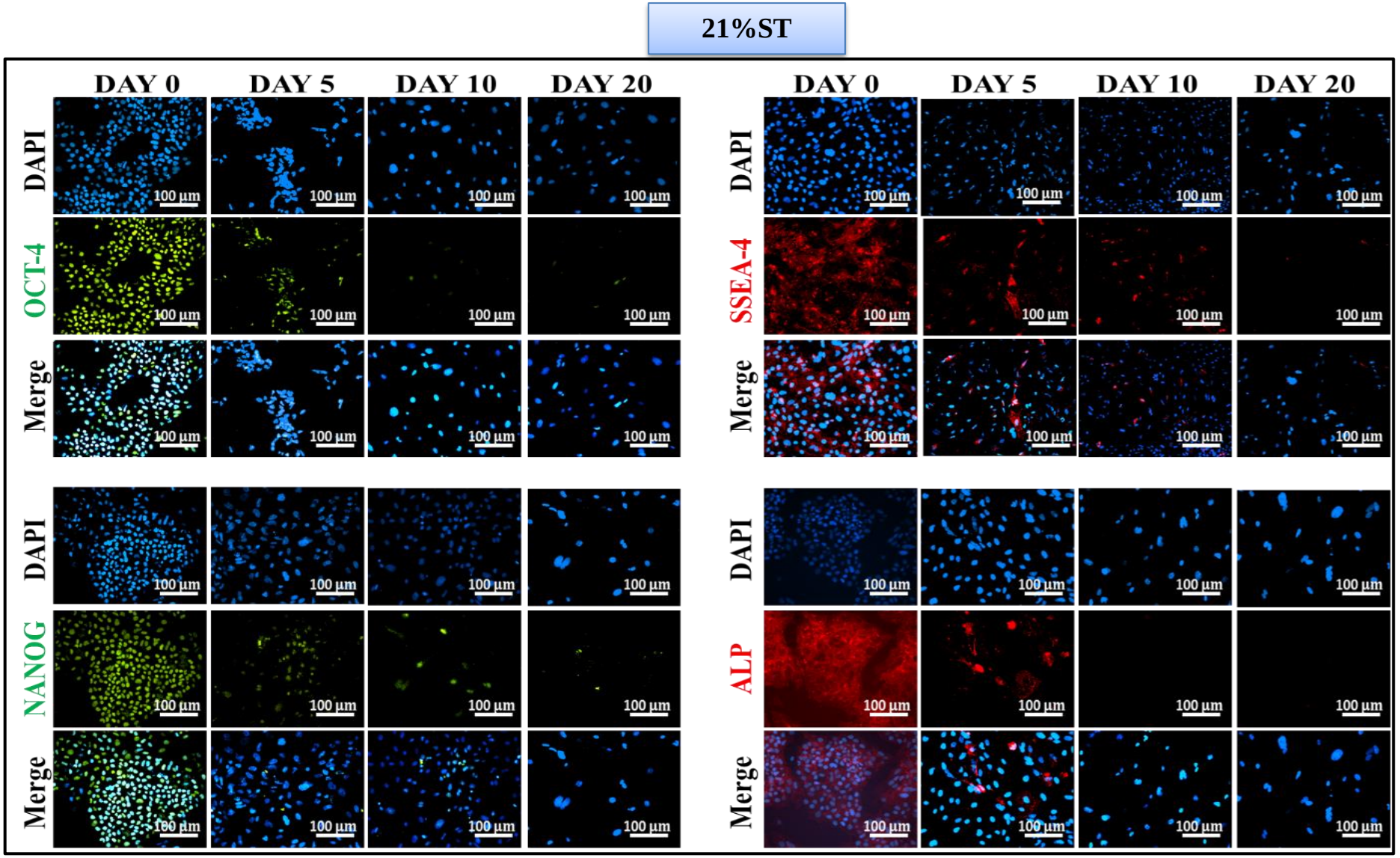
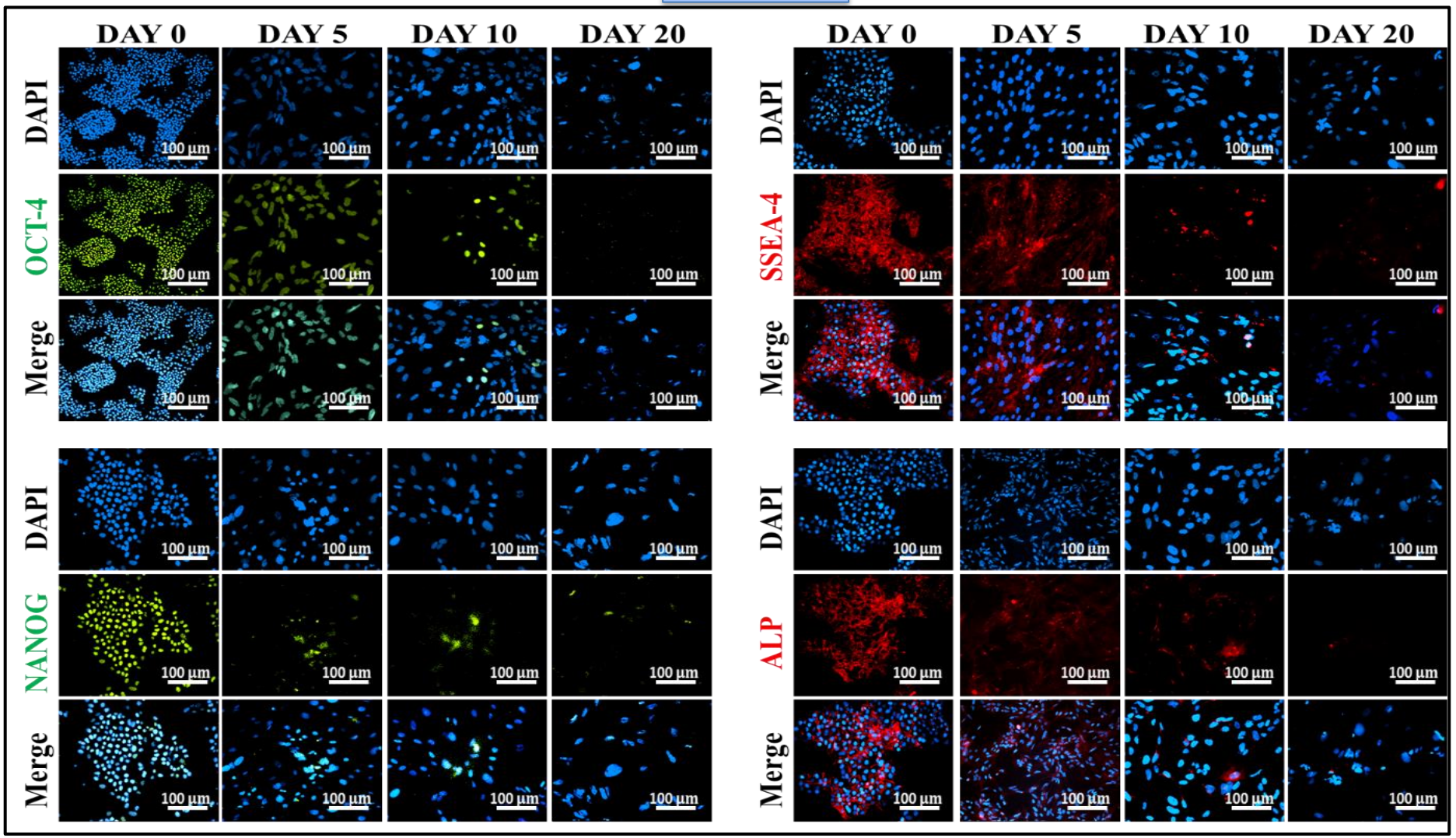


Figure 4.7 Immunofluorescence staining for pluripotency markers in undifferentiated and differentiating SHEF2 cells. Cells were seeded in 24 well-plates in three different oxygen tensions at selected time points (0, 5, 10 and 20 days). Red and green colour indicates antigen, blue DAPI. Each scale bar represents 100 µm.


21%ST
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm


2%PG
DAY 0
DAY 5
DAY 10
DAY 20
DAPI
OCT-4
Merge
SSEA-4
NANOG
ALP
100 µm

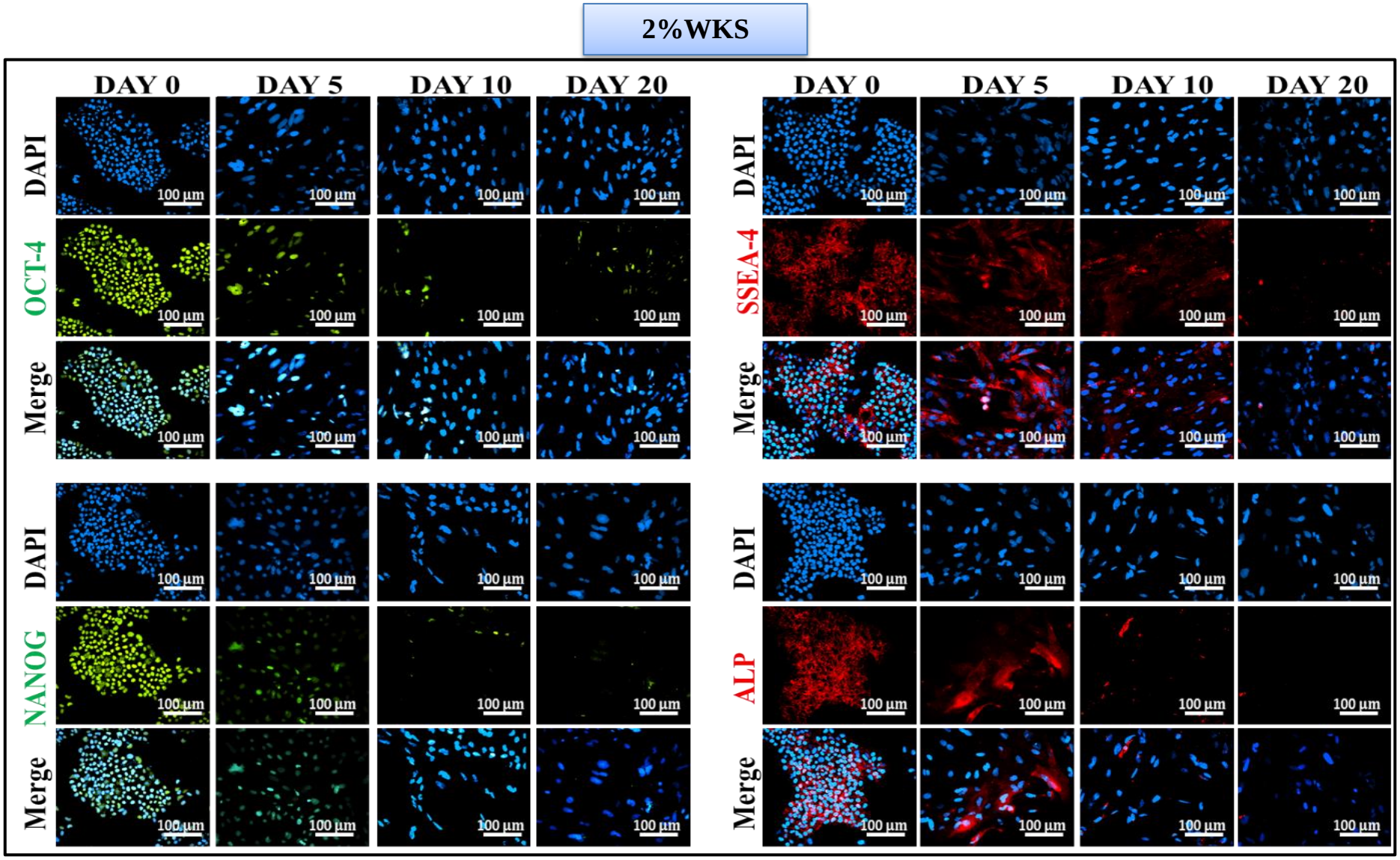


Figure 4.8 Immunofluorescence staining for pluripotency markers in undifferentiated and differentiating hiPSC line (ZK2012L) cells. Cells were seeded in 24 well-plates in three different oxygen tensions at selected time points (0, 5, 10 and 20 days). Red and green colour indicates antigen, blue DAPI. Each scale bar represents 100 µm.

4.4.5 Reduced oxygen increased core pluripotency factors OCT-4 and SOX-2 in undifferentiated hPS cells

hPS cell self-renewal and differentiation are regulated by a core group of genes known as pluripotency transcription factors such as OCT-4, NANOG and SOX-2 (Boyer et al., 2005; Wang et al., 2012). To identify whether the expression of this group of genes are directly regulated by oxygen at transcriptional level we isolated RNA from hPS cells and their differentiated cells cultured at three different oxygen conditions (21%ST, 2%PG and 2%WKS). OCT-4 levels were increased in undifferentiated SHEF1, SHEF2 and ZK2012L in both reduced oxygen conditions (2%PG and 2%WKS) and significantly so in 2%PG and 2%WKS in ZK2012L (1.84 ± 0.20 and 1.64 ±0.21, respectively) compared with air oxygen **(Figure 4.9)**. In contrast, the level of OCT-4 was decreased in day 5, 10 and 20 after differentiation following treatment with 2%PG and 2%WKS but no significant differences were noticed except the cells that cultured in 2%PG for 20 days in SHEF1 and SHEF2 (0.33 ± 0.28 and 0. 0.30 ± 0.16, respectively), and 2%WKS in SHEF2 (0.38 ± 0.20) compared with air oxygen **(Figure 4.9)**. The level of NANOG was decreased in undifferentiated SHEF1, SHEF2 and ZK2012L cells following treatment with 2%PG and 2%WKS but no significant differences were noticed except the cells that cultured in 2%PG in SHEF1 (0.51 ± 0.31), in comparison to those cultured in 21%ST **(Figure 4.9)**. NANOG expression was increased in differentiated SHEF1, SHEF2 and ZK2012L cells grown in reduced oxygen conditions following treatment with reduced oxygen at day 10 and 20. Furthermore, Similar increased was observed in SOX-2 level in undifferentiated SHEF1, SHEF2 and ZK2012L cells and their differentiated cells that cultured under hypoxia conditions (2%PG and 2%WKS), in comparison to those cultured in 21%ST **(Figure 4.9)**.

The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was a significant increase in the OCT-4 expression in cells cultured under 2%WKS (1.64 ± 0.13) in comparison to those cultured in air oxygen (21%ST) **(Figure 4.9)**. In contrast, the level of OCT-4 was decreased in day 5, 10 and 20 after differentiation following treatment with 2%PG and 2%WKS but no significant differences were noticed in comparison to cells cultured in air oxygen. As shown in **Figure 4.9**. The level of NANOG was decreased significantly in undifferentiated cells following treatment with 2%PG and 2%WKS (0.57 ± 0.1 and 0.64 ± 0.11, respectively), and in day 5 after differentiation following treatment with 2%PG (0.63 ± 0.09) in comparison to those cultured in air oxygen (21%ST). Also, the results showed increased in expression of SOX-2 in cells cultured under reduced oxygen conditions (2%PG and 2%WKS), but there were no significant differences was observed except the cells that cultured in undifferentiated 2%WKS (2.0 ± 0.4) in comparison to those cultured in air oxygen (21%ST) **(Figure 4.9)**.

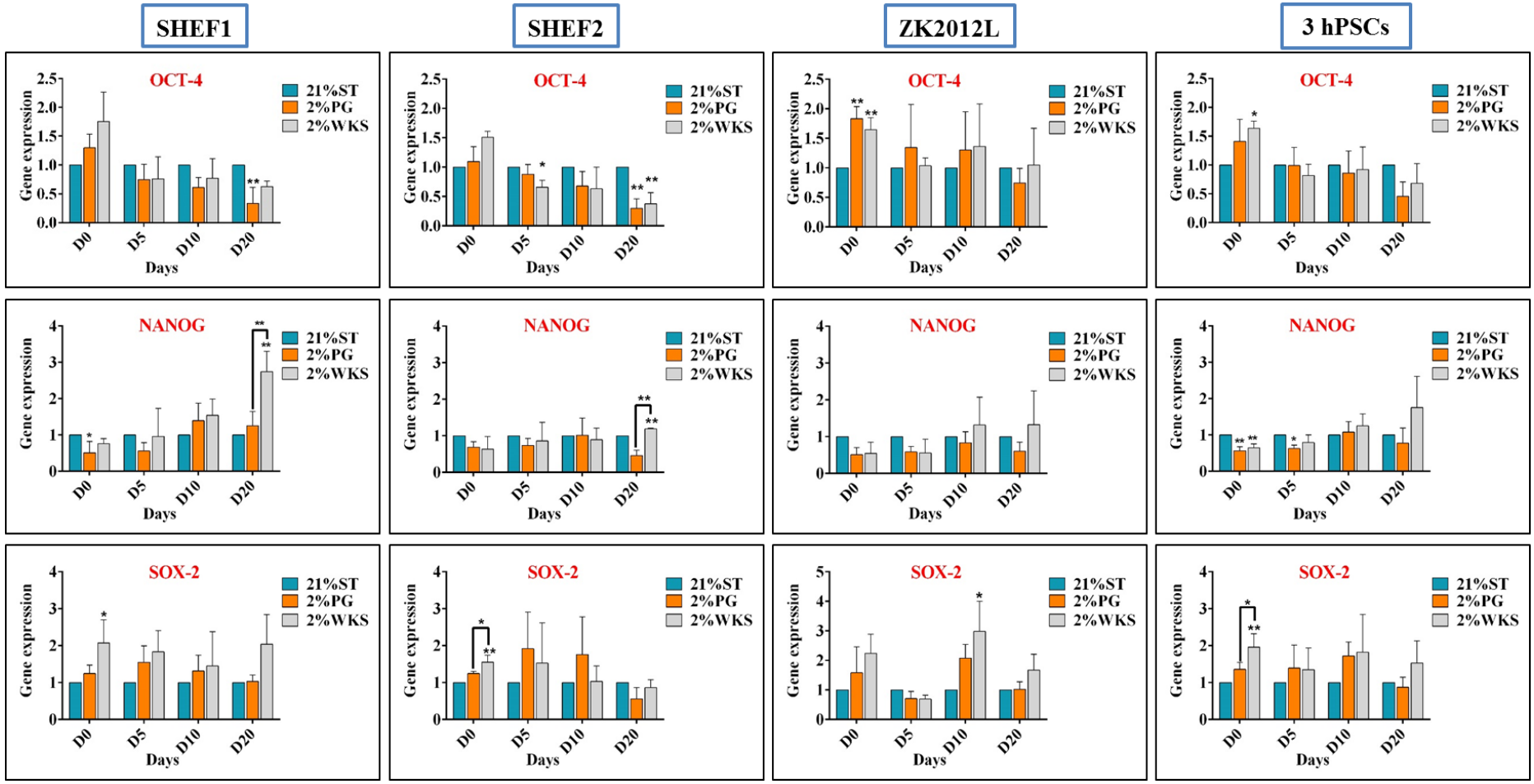


Figure 4.9 Quantitative RT-PCR analysis of OCT-4 (POU5F1), NANOG, and SOX-2 in response to reduce oxygen in three cell lines of hPS cells. The expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{-\Delta\Delta Ct}$ of treated cell to untreated cell. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.4.6 Reduced oxygen induces DNA hypomethylation in hPS cells and their differentiated progeny

To examine the global DNA methylation (5mC and 5hmC) patterns in undifferentiated hPS cells and their differentiated progeny DNA was isolated from both undifferentiated hPS cells (day 0) and following 5, 10 and 20 days of differentiation in 21%ST, 2%PG and 2%WKS. This DNA was then explored using an immunospecific method to determine global levels of 5mC and 5hmC. A decreased level of global DNA methylation in the total DNA extracted from all three pluripotent stem cells cultured and their differentiated progeny in both reduced oxygen conditions (2%PG and 2%WKS) in comparison to oxygen air (21%ST) was noted **(Figure 4.10 and 4.11)**.

Also, we noted that the level of global DNA methylation in spontaneous differentiation cells was decreased in a time-dependent manner. The level of 5mC in SHEF1 cells was decreased significantly in undifferentiated cells following incubation in 2%PG (0.82 ± 0.06, $p<0.05$) and 2%WKS (0.73 ± 0.07, $p<0.01$) in comparison to those cultured in 21%ST (1.01 ± 0.041), and in day 20 from spontaneous differentiation in 2%WKS (0.52 ± 0.09, $p<0.05$) whilst air oxygen was higher (0.72 ± 0.074) **(Figure 4.10A)**. Also, as shown in **Figure 4.10B** DNA methylation decreased significantly in undifferentiated SHEF2 cells grown in 2%PG, a by percentage change in of 5-methylcytosine (0.74 ± 0.03, $p<0.05$), and in day 20 (0.51 ± 0.02, $p<0.05$) after differentiation versus 21%ST (0.84 ± 0.05 and 0.70 ± 0.08), respectively. SHEF2 cells cultured in 2%WKS showed significant reduction in 5mC content in differentiation cells in day 10 (0.50 ± 0.03, $p<0.01$) and 20 ($0.4\ 1 \pm 0.06$, $p<0.01$). hiPSC line (ZK2012L) showed significant decreases <0.05, in global 5mC level when cultured in 2%PG and 2%WKS oxygen excepting day 10 from differentiation **(Figure 4.10C)**. Also, the results of three hPS cells (SHEF1, SHEF2 and hiPSCs) together showed decreased global 5mC level in cells cultured under reduced oxygen conditions (2%PG and 2%WKS), but no

significant differences were observed except for the cells that were cultured in 2%WKS at day 20 from differentiation (0.46 ± 0.5) in comparison to those cultured in air oxygen (0.69 ± 0.5) **(Figure 4.10D)**.

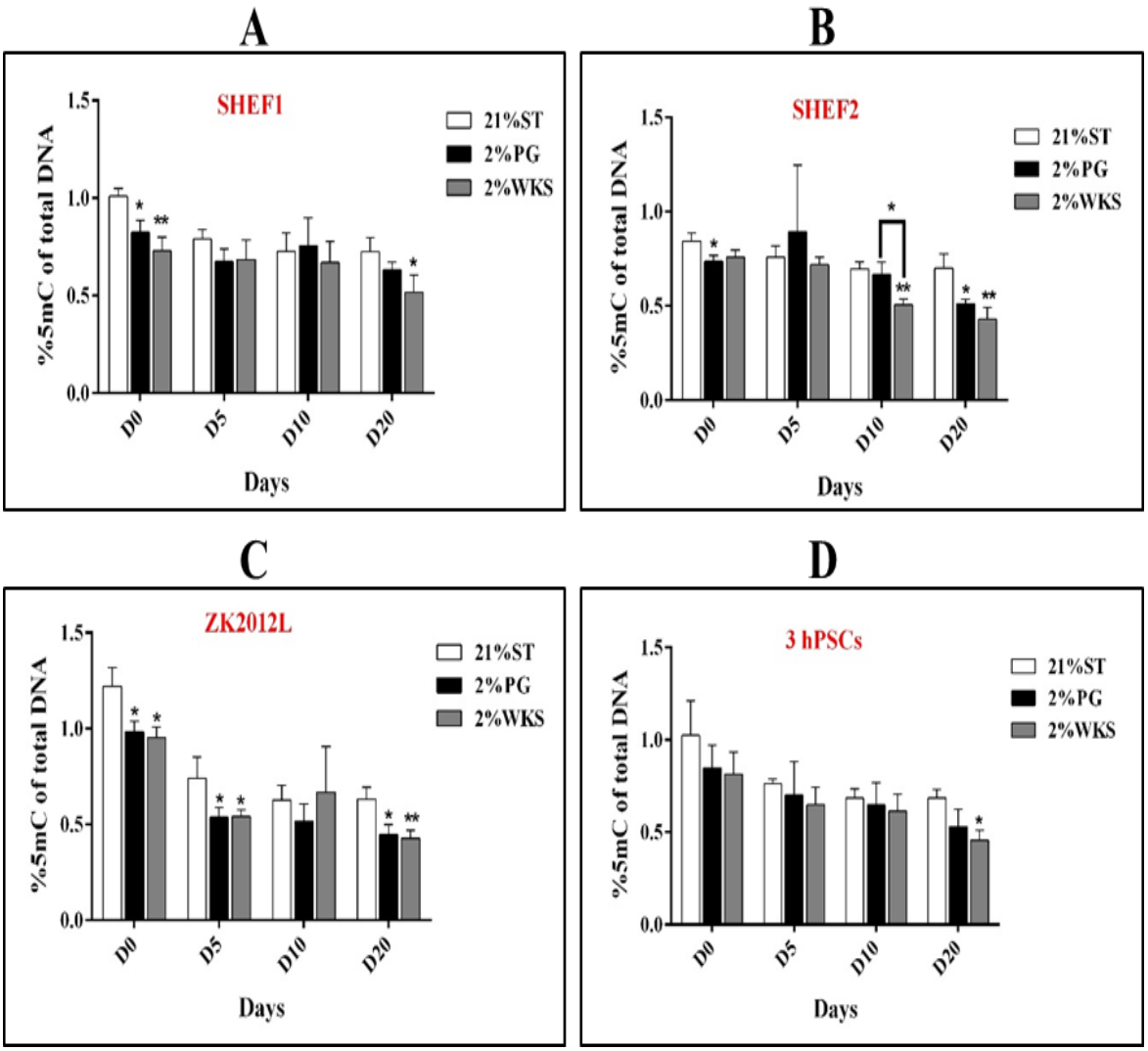


Figure 4.10 Percentage of 5mC in the total DNA extracted from hPS cells in response to reduce oxygen conditions. A) SHEF1 B) SHEF2 C) hiPSC line (ZK2012L) D) 3 hPSC line (SHEF1, SHEF2 and ZK2012L). hPS cells were incubated in air oxygen (21%ST) and normoxia physiological conditions (2%PG and 2%WKS) and the methylated DNA fraction in the total isolated DNA was measured using MethylFlash™ Methylated DNA quantification kit. Y-axis indicates 5-methylcytosine absorbance (450 nm). X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Similar significant reductions were observed in global 5hmC content in SHEF1 cells grown under reduced oxygen conditions 0.08 ± 0.006, $p<0.05$ and 0.06 ± 0.008, $p<0.01$ in day 0 (2%PG and 2%WKS, respectively), and in day 10 and 20 in 2%WKS from differentiation (0.05 ± 0.01, $p<0.05$ and 0.04 ± 0.008, $p<0.05$), respectively, in comparison to those cultured in 21%ST **(Figure 4.11A)**. In contrast, the presence of reduced oxygen did not significantly impact on 5hmC level in SHEF2 cells except the cells that cultured in 2%WKS in undifferentiation status (0.14 ± 0.02, $p<0.05$) **(Figure 4.11B)**. On the other hand, there was a significant decrease ($p<0.05$) in the percentage of 5hmC in the total DNA extracted from hiPSCs cells cultured in 2%WKS conditions at day 0, 5 and 20 (0.13 ± 0.02; 0.06 ± 0.02 and 0.057 ± 0.009), respectively, and in day 20 in 2%PG (0.06 ± 0.01) in comparison to those cultured in 21% ST **(Figure 4.11C)**. Also, the results of three hPS cells (SHEF1, SHEF2 and hiPSCs) together showed decreased global 5hmC level in cells cultured under reduced oxygen conditions (2%PG and 2%WKS), but there were no significant differences was observed in comparison to those cultured in 21% ST **(Figure 4.11D)**.

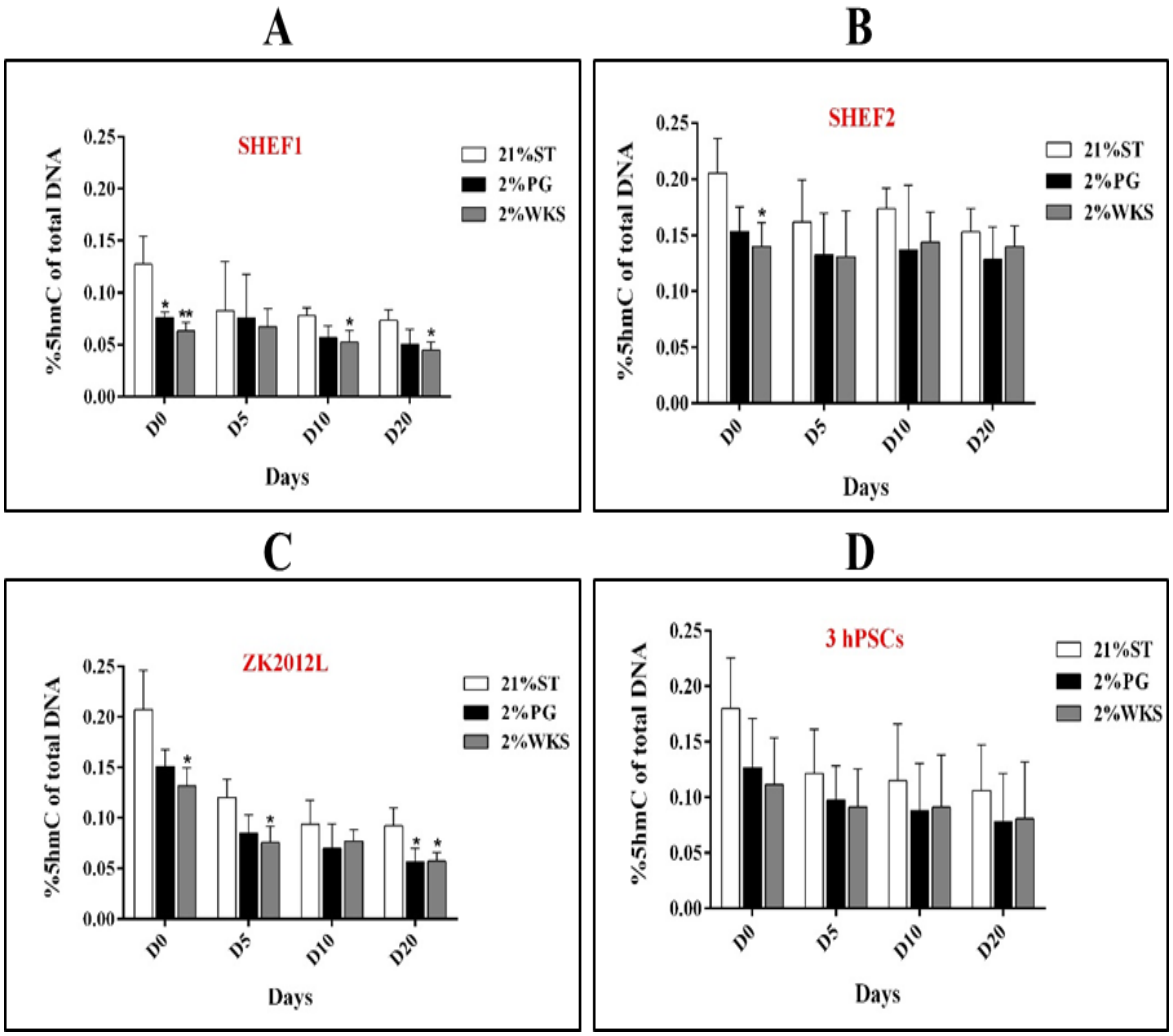


Figure 4.11 Percentage of 5hmC in the total DNA extracted from hPS cells in response to reduce oxygen conditions. A) SHEF1 B) SHEF2 C) hiPSC line (ZK2012L) D) 3 hPSC line (SHEF1, SHEF2 and ZK2012L). hPS cells were incubated in air oxygen (21%ST) and normoxia physiological conditions (2%PG and 2%WKS) and the methylated DNA fraction in the total isolated DNA was measured using MethylFlash^{TM} hydroxymethylated DNA quantification kit. Y-axis indicates 5-hydroxymethylation absorbance (450 nm). X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.4.7 Expression of DNMTs and TETs genes by hPS Cells and their differentiated progeny in response to low oxygen level

RT-qPCR was employed to examine whether the reduction in global DNA methylation (5mC and 5hmC) in response to physiological normoxia were in relation to changes in the expression of DNMTs/TETs genes. The results of SHEF1 cells showed there was no significant change in the relative expression of DNMT1, DNMT3A, TET2 and TET3 excepting DNMT3A and TET3 expression in day 5 from differentiation in 2%WKS conditions (0.35 and 0.44, respectively), and in day 20 in 2%PG and 2%WKS (0.35 and 0.43, respectively) in TET3 in comparison to those cultured in 21% ST **(Figure 4.12)**. The level of DNMT3B was decreased significantly in undifferentiated cells following treatment with 2%PG and 2%WKS (0.37 and 0.52, respectively), and in all time course from spontaneous differentiation in 2%WKS (0.55, 0.50 and 0.51, respectively) **(Figure 4.12)**. Similar significant reduction was observed in TET1 level in undifferentiated SHEF1 cells grown under 2%WKS conditions (0.51), and in day 5, 10 and 20 in 2%WKS from differentiation (0.54, 0.66 and 0.59, respectively). The level of DNMT3L was decreased significantly in undifferentiated cells following treatment with 2%PG and 2%WKS (0.49 and 0.65, respectively), and in day 5 and day 20 from spontaneous differentiation in 2%WKS (0.55 and 0.67, respectively), and in day 10 in 2%PG and 2% WKS (0.48 and 0.53, respectively) in comparison to those cultured in 21% ST **(Figure 4.12)**.

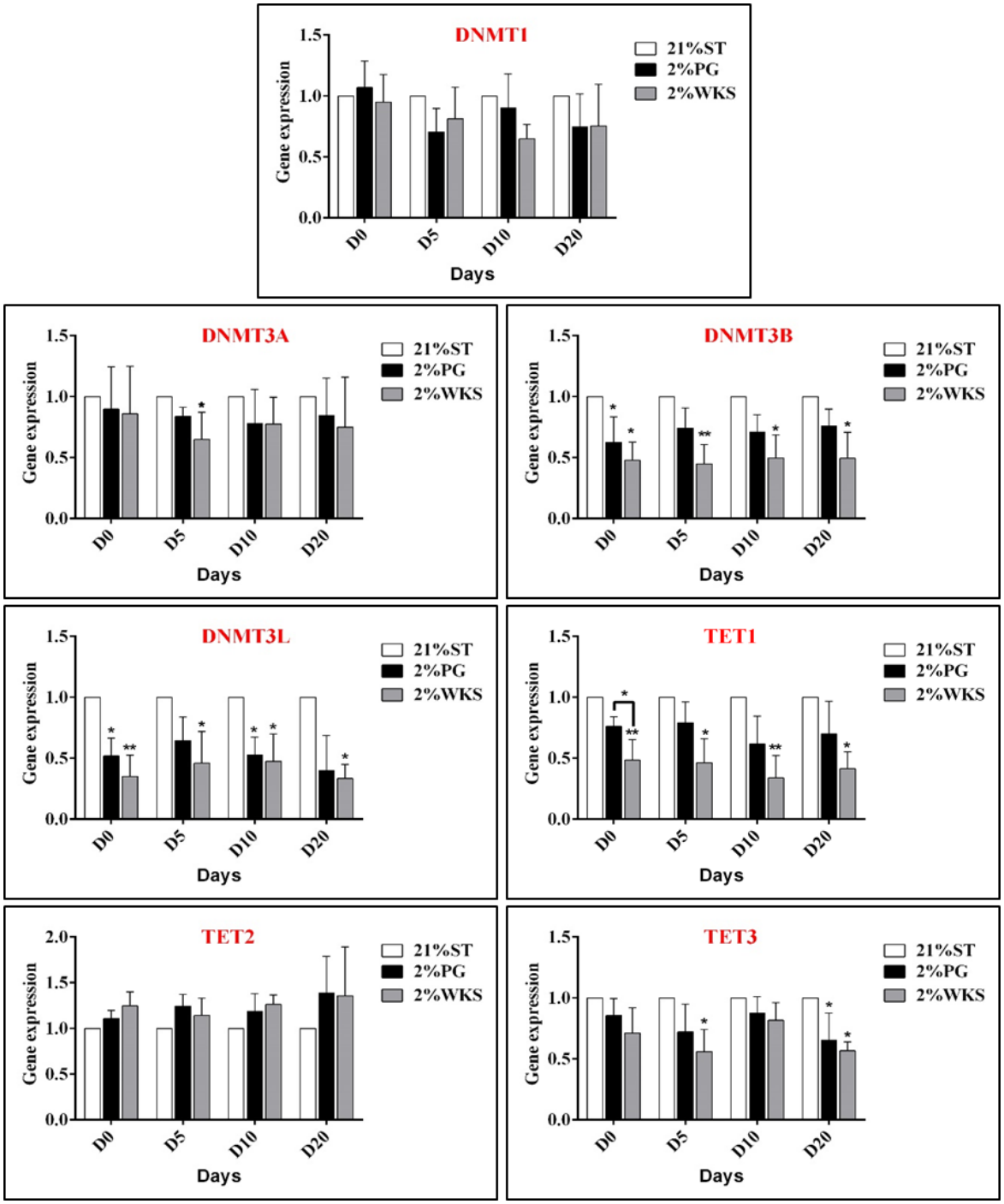


Figure 4.12 The RT-qPCR expression of the DNMTs and TETs after SHEF1 cells incubation under different oxygen level. RNA quantification of DNMTs and TETs isolated from SHEF1 cells following incubation in air oxygen (21%ST) and normoxia physiological culturing conditions (2%PG and 2%WKS). The expression was normalized to the expression of β-actin in the SHEF1 cells. Y-axis indicates relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to untreated cell. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Consistent results were observed for SHEF2 cells treated with physiological normoxia. Under 2%PG and 2%WKS significant reduction in DNMT3B expression was noticed in undifferentiated cells (0.44 and 0.22, respectively), and after 10 days from differentiation (0.49 and 0.64, respectively), and day 20 in 2%WKS (0.84) in comparison to those cultured in 21% ST **(Figure 4.13)**. TET1 expression decreased in a pattern similar to that of DNMT3B expression. Furthermore, a significant decrease in the expression of DNMT3L was showed under 2%WKS conditions in undifferentiated cells (0.52), and after 20 days in 2%PG and 2%WKS (0.40 and 0.72, respectively) **(Figure 4.13)**. No significant change was noted in the expression of DNMT1, DNMT3A, TET2 and TET3 excepting DNMT3A and TET3 expression in undifferentiation cells and in day 20 from differentiation in 2%WKS conditions (0.26 and 0.70, respectively) in comparison to those cultured in 21% ST **(Figure 4.13)**.

Similar significant reduction was observed in DNMT3B level in undifferentiated iPS cells grown under 2%PG and 2%WKS conditions (0.50 and 0.55, respectively), and in day 5 (0.34 and 0.59, respectively) and day 20 (0.37 and 0.45, respectively) from differentiation in comparison to those cultured in 21% ST **(Figure 4.14)**. The level of TET1 was decreased significantly in undifferentiated cells and day 10 after differentiation following treatment with 2%WKS (0.35 and 0.43, respectively), and in day 20 in 2%PG and 2%WKS conditions (0.13 and 0.4, respectively) **(Figure 4.14)**. Significant decrease in the expression of DNMT3L was showed under 2%PG and 2%WKS conditions in undifferentiated cells (0.61 and 0.59, respectively), and after 20 days (0.62 and 0.61, respectively) **(Figure 4.14)**. No significant change in the expression of DNMT1, DNMT3A and TET2 excepting DNMT3A and DNMT1 expression in undifferentiation cells and day 5 from differentiation in 2%WKS conditions (0.36 and 0.48, respectively) in comparison to those cultured in 21% ST **(Figure**

4.14). In addition, TET3 expression increased significant only in undifferentiated cells in both reduced oxygen 2%PG and 2%WKS conditions (0.44 and 0.46, respectively) in comparison to those cultured in 21% ST **(Figure 4.14)**.

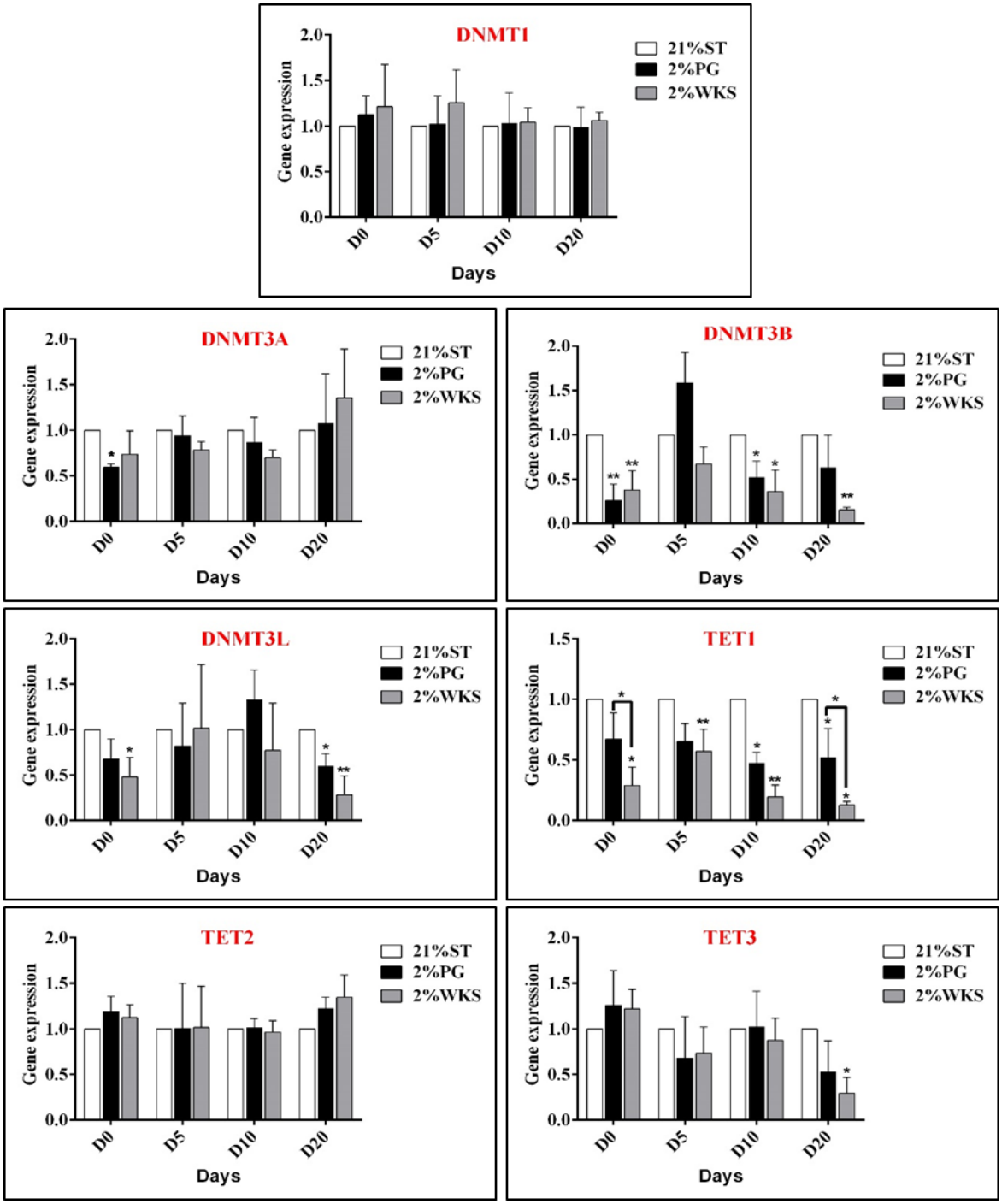


Figure 4.13 The RT-qPCR expression of the DNMTs and TETs after SHEF2 cells incubation under different oxygen level. RNA quantification of DNMTs and TETs isolated from SHEF2 cells following incubation in air oxygen (21%ST) and normoxia

physiological culturing conditions (2%PG and 2%WKS). The expression was normalized to the expression of β-actin in the SHEF2 cells. Y-axis indicates relative changes in $2^{-\Delta\Delta Ct}$ of treated cell to untreated cell. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

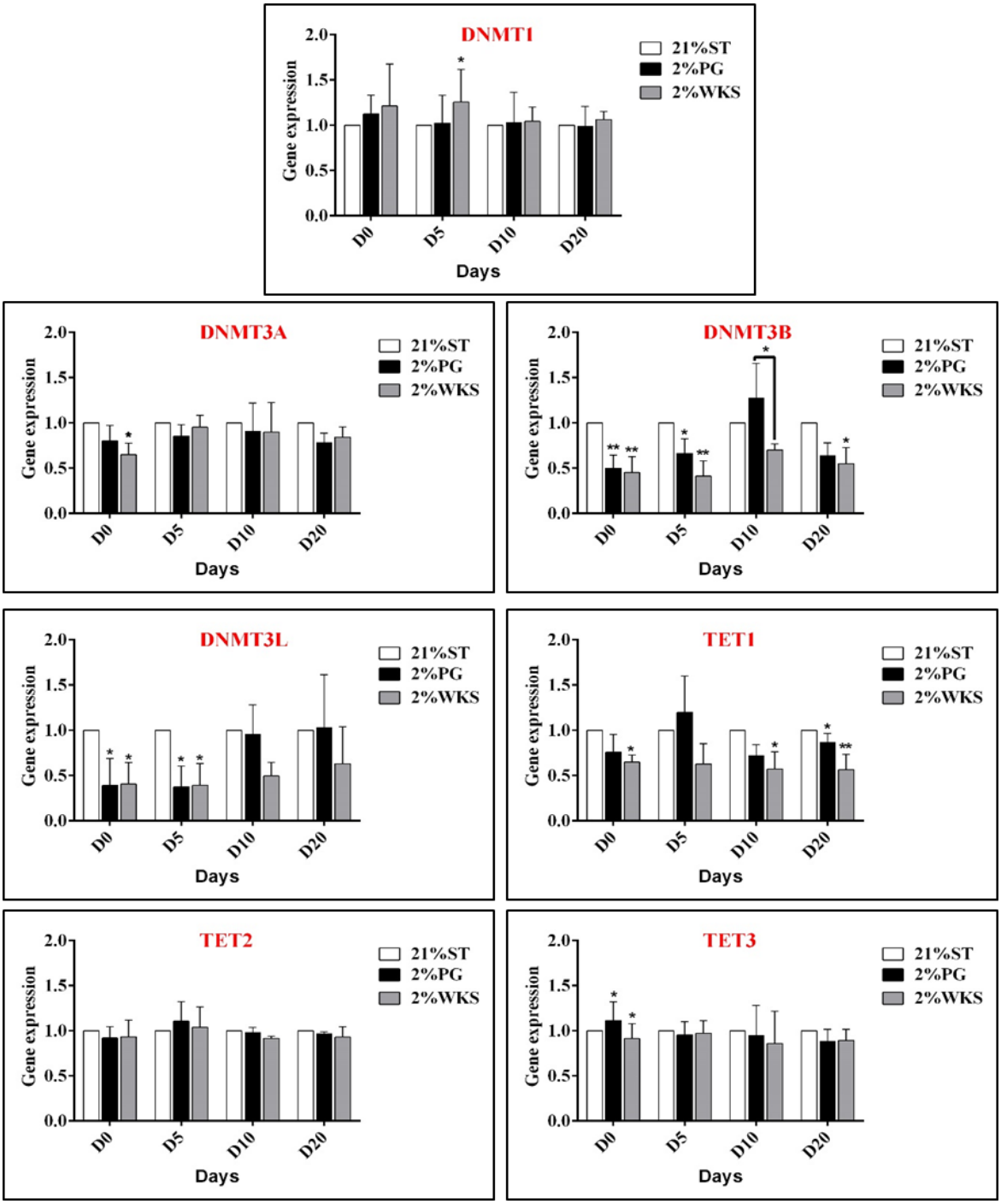


Figure 4.14 The RT-qPCR expression of the DNMTs and TETs after hiPSC line (ZK2012L) cells incubation under different oxygen level. RNA quantification of DNMTs and TETs isolated from ZK2012L cells following incubation in air oxygen (21% ST) and h normoxia physiological culturing conditions (2%PG and 2%WKS). The expression was

normalized to the expression of β-actin in the ZK2012L cells. Y-axis indicates relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to untreated cell. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was a significant reduction in DNMT3B level in undifferentiated cells grown under 2%PG and 2%WKS conditions (0.46 and 0.44, respectively), and in day 20 (0.67 and 0.40, respectively) from differentiation in comparison to those cultured in 21% ST **(Figure 4.15)**.The level of TET1 was decreased significantly in undifferentiated cells following treatment with 2%WKS (0.48), and in day 5, 20 from differentiation (0.0.55 and 0.57, respectively), and in day 10 in 2%PG and 2%WKS (0.60 and 0.37, respectively) (**Figure 4.15**). Significant decrease in the expression of DNMT3L was showed under 2%PG and 2%WKS conditions in undifferentiated cells (0.53 and 0.41, respectively), and after 20 days following treatment with 2%WKS (0.41). No significant change in the expression of DNMT1, DNMT3A and TET2 excepting DNMT3A expression at day 10 from differentiation in 2%WKS conditions (0.79) was observed in comparison to those cultured in 21% ST **(Figure 4.15)**. In addition, TET3 expression increased significant only in differentiated cells in 2%PG and 2%WKS conditions at day 5 (0.69 and 0.61, respectively), and day 20 (0.62 and 0.49, respectively), and after 10 days following treatment with 2%WKS (0.80) in comparison to those cultured in 21% ST **(Figure 4.15)**.

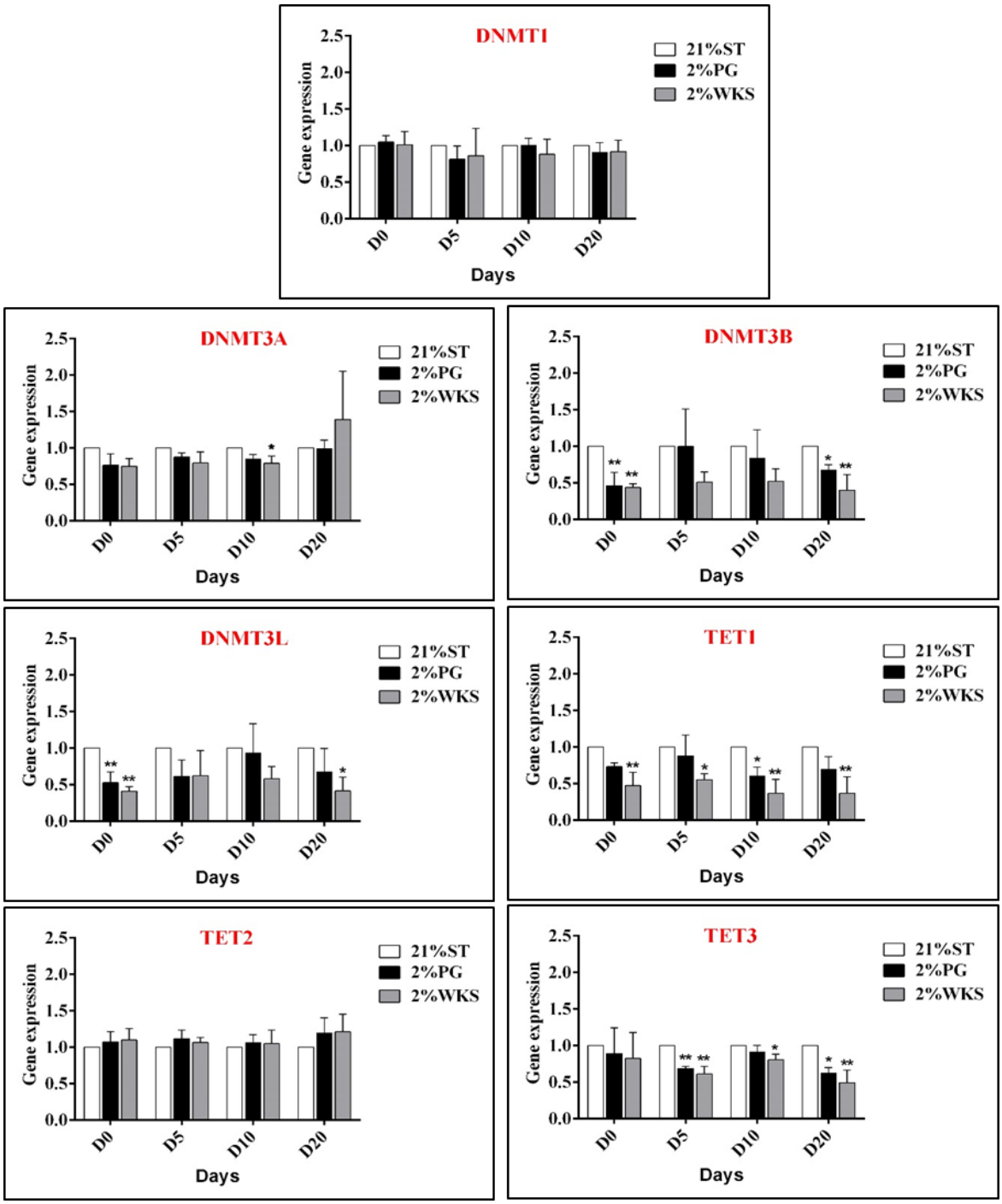


Figure 4.15 The RT-qPCR expression of the DNMTs and TETs after 3 hPSC line (SHEF1, SHEF2 and ZK2012L) cells incubation under different oxygen level. RNA quantification of DNMTs and TETs isolated from 3 hPSC line following incubation in air oxygen (21% ST) and normoxia physiological culturing conditions (2%PG and 2%WKS). The expression was normalized to the expression of β-actin in the ZK2012L cells. Y-axis indicates relative changes in $2^{\wedge}\Delta\Delta^{Ct}$ of treated cell to untreated cell. X-axis indicates time

(days). Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.4.8 Immunoblotting assay demonstrated reduced DNMT3B and TET1 protein expression

To confirm that the decrease in DNMT3B and TET1 transcription was accompanied by down-regulation of DNMT3B and TET1 at protein level, this was assessed using western blot analysis on protein isolated from undifferentiated hPS cells and their differentiated progeny cultured at day 20 in normoxia physiological conditions compared to cells maintained in air oxygen conditions. Profiling protein expression showed that there was a downregulation of DNMT3B and TET1 in hPS cells and their differentiated progeny cultured at day 20 in reduced oxygen conditions in a pattern similar to that of mRNA expression in hPS cell lines **(Figure 4.16)**.

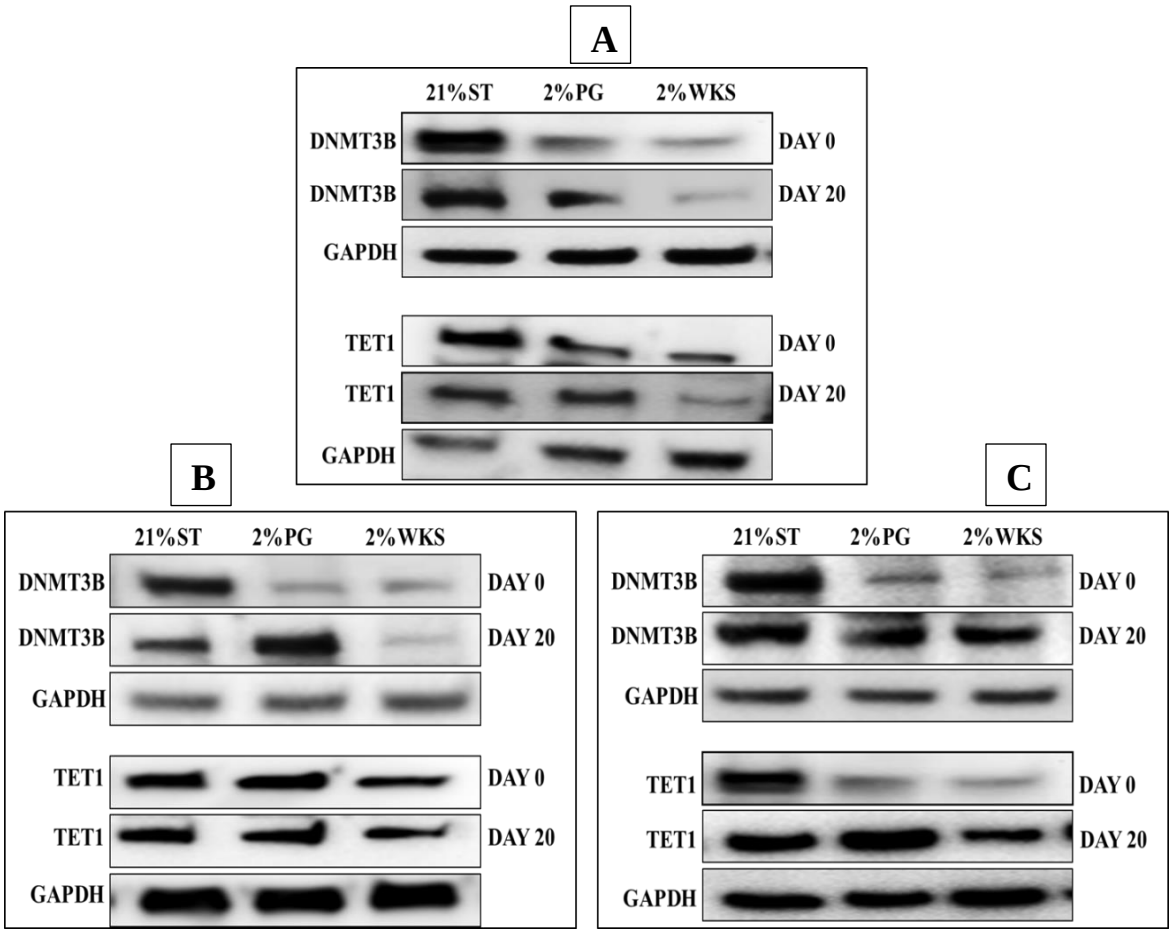


Figure 4.16 Changes in DNMT3B and TET1 expression in hPSCs, at protein levels measured by immunoblotting. A) SHEF1 B) SHEF2 C) hiPSC line (ZK2012L). Protein was isolated from undifferentiated hPS cells and their differentiated progeny cultured at day 20 following incubation in air oxygen (21%ST) and normoxia physiological culturing conditions (2%PG and 2%WKS). GAPDH was used as a control.

4.4.9 Gene-specific promoter methylation in hPS cells and their differentiated progeny

Section 4.4.6 showed there were differences in the mean levels of DNA methylation between reduced oxygen and air oxygen conditions in hPS cells and their differentiated progeny. Also, the results showed that low oxygen induced the changes in hPS cells and their differentiated progeny at both transcript and protein levels **(4.4.7 and 8)**. To confirmed that these changes were consistent with methylation at promoter level, DNA methylation status of seven genes promoter at CpGs levels was determined using pyrosequence assay. The

results of SHEF1 cells showed there were a significant increase in the mean level of DNMT3B methylation in undifferentiated cells following treatment with 2%PG and 2%WKS (23% and 32%, respectively) compared with the air oxygen (11%), and in day 5 and 20 from spontaneous differentiation in 2%PG and 2%WKS (23% and 25%, respectively) compared with the air oxygen (13% and 11%, respectively) **(Figure 4.17)**. Also, there was a significant difference in the mean level of DNMT3B methylation between 2%PG and 2%WKS in undifferentiated cells and day 20 from spontaneous differentiation. The level of DNMT3L methylation was increased significantly in undifferentiated cells and in day 10 after differentiation following treatment with 2%WKS (86% and 89%, respectively) compared with the air oxygen (71% and 75%, respectively), and in day 20 in 2%PG and 2%WKS conditions (85% and 90%, respectively) compared with the air oxygen (73%) **(Figure 4.17)**. By contrast, there was no significant change in the methylation level of DNMT1, DNMT3A, TET1, TET2 and TET3 genes was observed **(Figure 4.17)**.

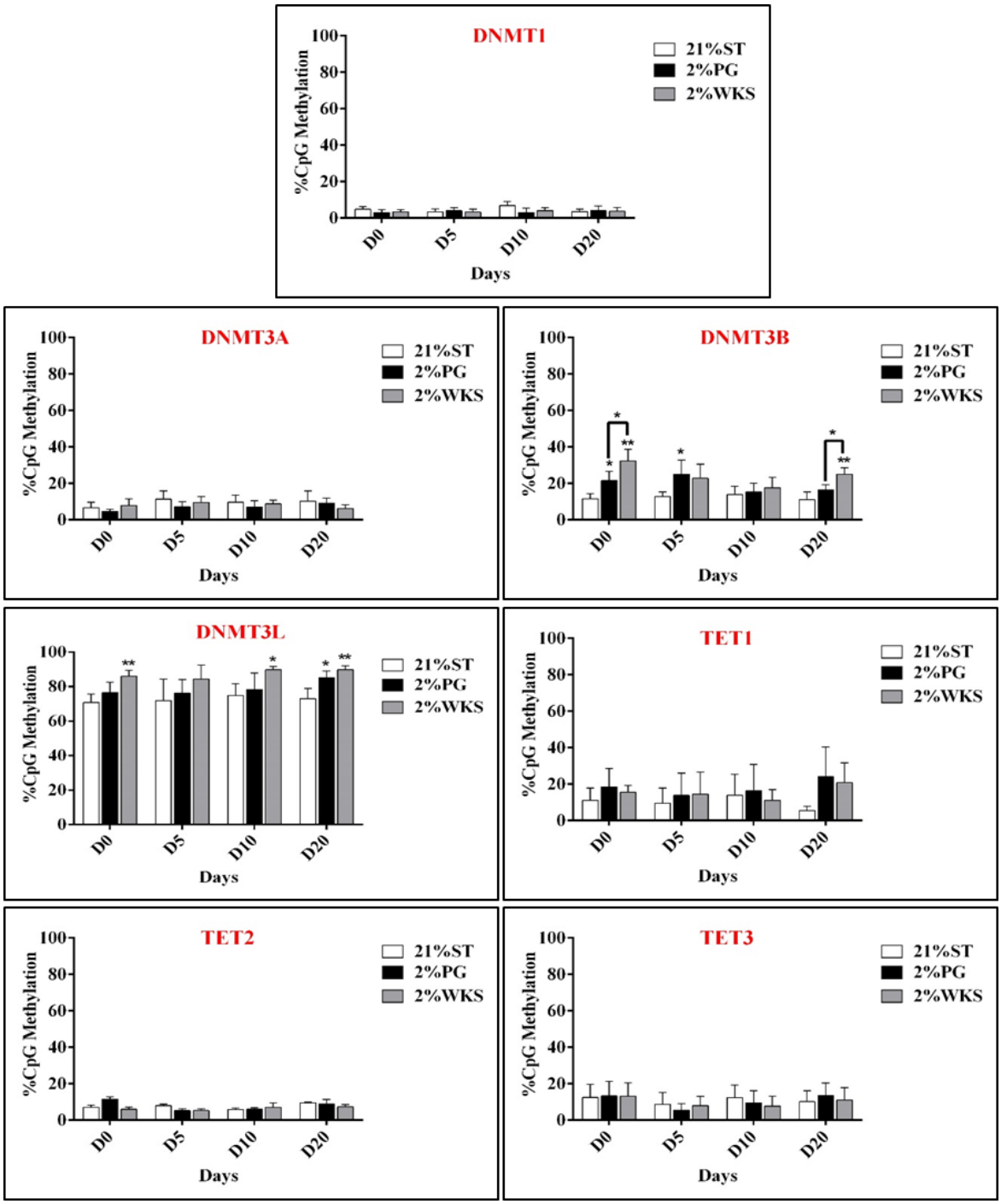


Figure 4. 17 Mean levels of methylation in physiological normoxia relative to air oxygen in SHEF1 cells. A set of seven genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01 vs air oxygen (21%ST).

Under 2%PG and 2%WKS a significant increase in DNMT3B methylation was noticed in undifferentiated SHEF2 cells (20% and 26%, respectively) compared with the air oxygen (9%), and after 5 and 20 days (20% and 27%, respectively) compared with the air oxygen (9% and 10%, respectively) **(Figure 4.18)**. Also, there was a significant difference in the mean level of DNMT3B methylation between 2%PG and 2%WKS in day 20 from spontaneous differentiation (15% and 27%, respectively). Furthermore, a significant increase in the methylation of DNMT3L was showed under 2%PG and 2%WKS conditions in undifferentiated cells (87% and 89%, respectively) compared with the air oxygen (68%), and after 20 days in 2%PG and 2%WKS (82% and 87%, respectively) compared with the air oxygen (70%). No significant change in the methylation of DNMT1, DNMT3A, TET1, TET2 and TET3 was observed **(Figure 4.18)**.

Similar significant increase was observed in the mean level of DNMT3B methylation in undifferentiated hiPSC line (ZK2012L) cells grown under 2%WKS conditions and in day 5, 10 and 20 from differentiation (26%, 19%, 13% and 25%, respectively) compared with the air oxygen, and in day 20 in 2%PG (5%, 4%, 5% and 8%, respectively), and in day 20 (28%) from differentiation **(Figure 4.19)**. Also, there was a significant difference in the mean level of DNMT3B methylation between 2%PG and 2%WKS in undifferentiated cells and day 5 and 10 from spontaneous differentiation. No significant change in the methylation of DNMT1, DNMT3A, DNMT3L, TET1, TET2 and TET3 was noticed **(Figure 4.19)**.

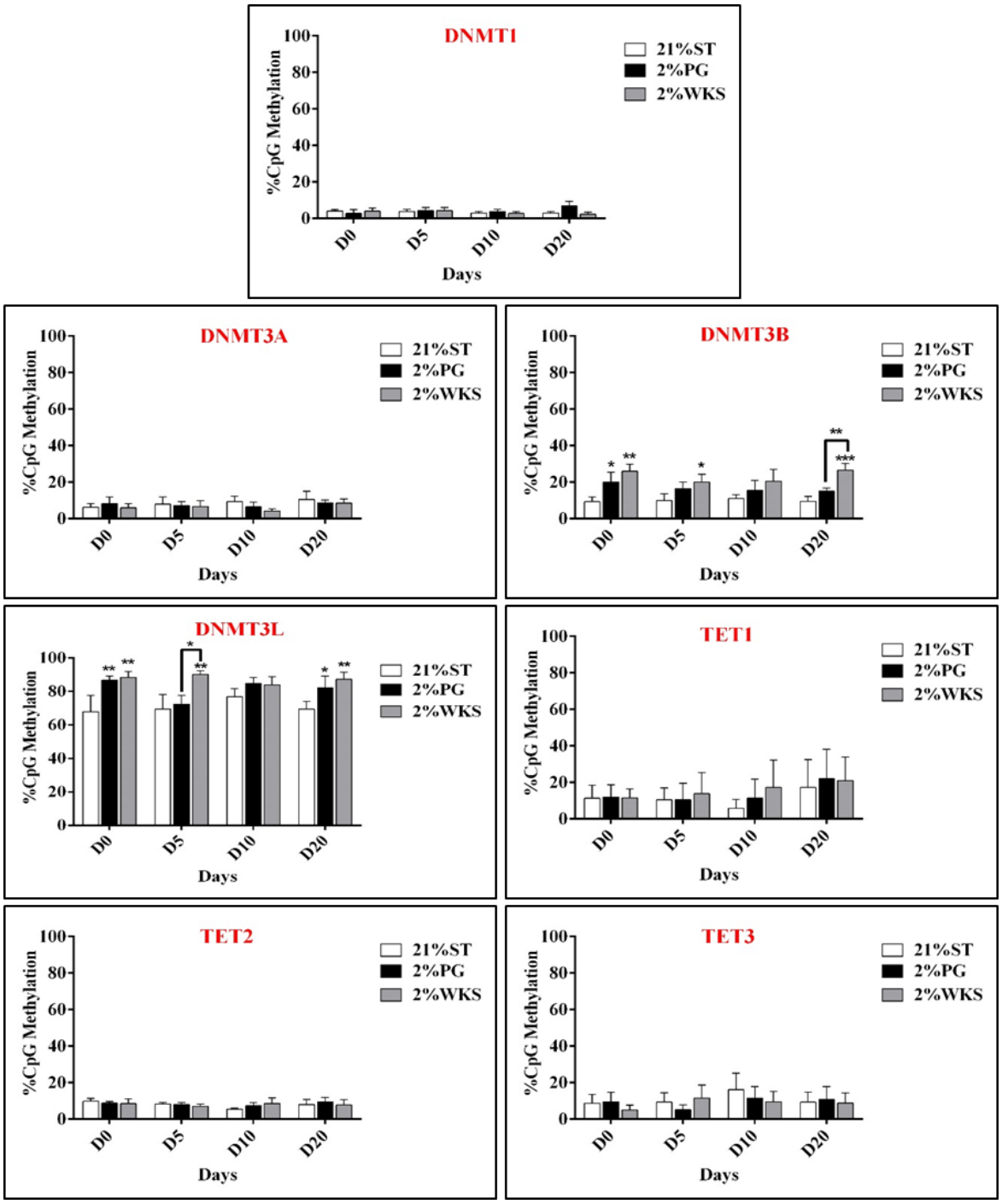


Figure 4.18 Mean levels of methylation in physiological normoxia relative to air oxygen in SHEF2 cells. A set of seven genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001 vs air oxygen (21%ST).

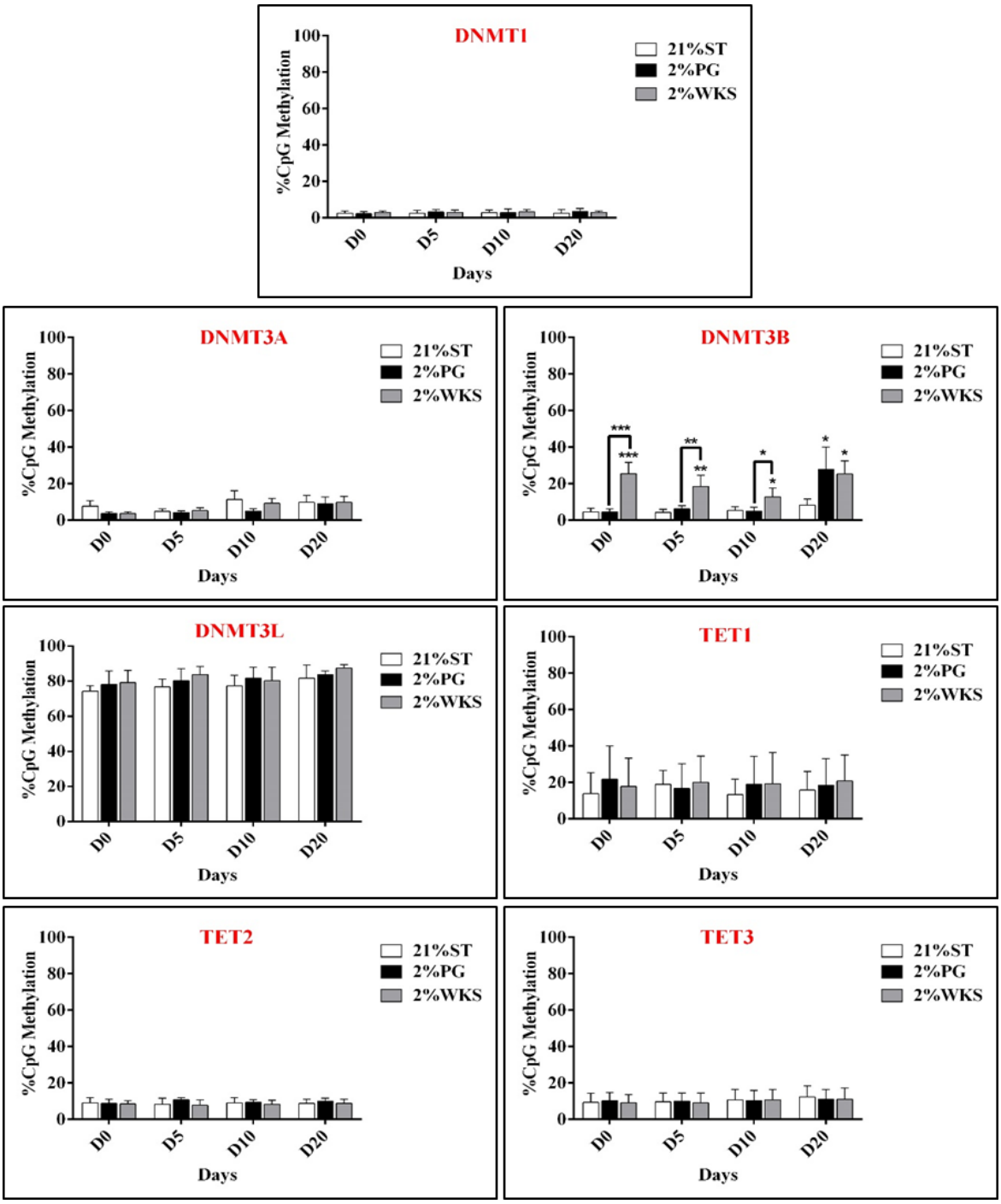


Figure 4.19 Mean levels of methylation in physiological normoxia relative to air oxygen in hiPSC line (ZK2012L) cells. A set of seven genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001 vs air oxygen (21%ST).

The data from all three hPS cells (SHEF1, SHEF2, and hiPSCs) was combined, and the results showed there was a significant increase in the mean level of DNMT3B methylation in undifferentiated cells grown under 2%WKS conditions and in day 20 from differentiation (28% and 26%, respectively) compared with the air oxygen **(Figure 4.20)**. Similar significant increase was observed in the mean level of DNMT3L methylation in undifferentiated cells grown under 2%WKS conditions and in day 5 and 20 from differentiation (85%, 86% and 88%, respectively). Furthermore, there was a significant difference in the mean level of DNMT3L methylation between 2%PG and 2%WKS at day 5 from spontaneous differentiation. No significant change in the methylation of DNMT1, DNMT3A, TET1, TET2 and TET3 was found **(Figure 4. 20)**.

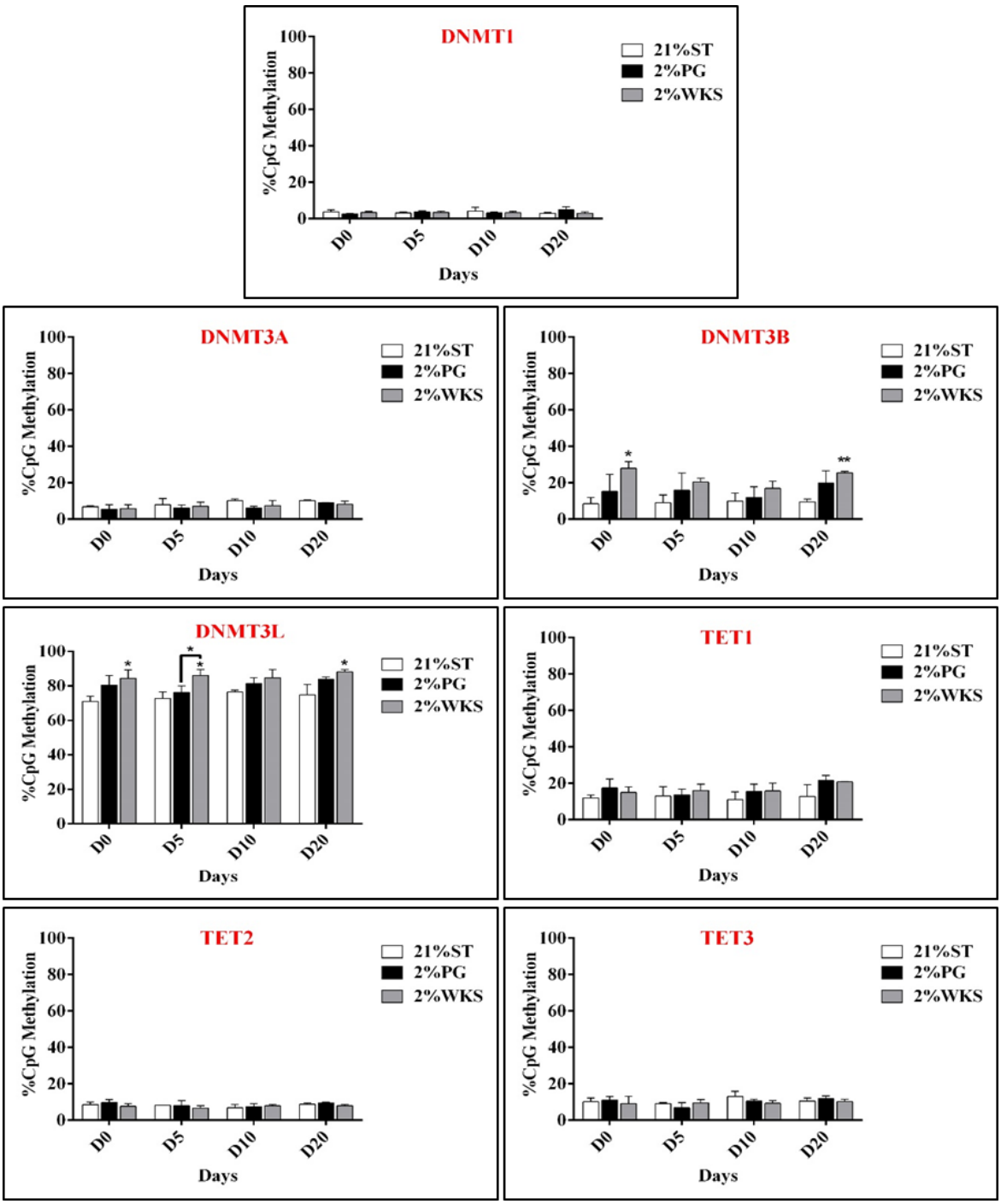


Figure 4.20 Mean levels of methylation in physiological normoxia relative to air oxygen in 3 hPSC line (SHEF1, SHEF2 and ZK2012L) cells. A set of seven genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates time (days). Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001 vs air oxygen (21%ST).

4.4.10 Reduced oxygen enriches HIF2A expression at protein level in hPS cells and their differentiated progeny

To identify the effects of reduced oxygen incubation on the HIFs gene expression profiles, transcriptional and protein levels of HIF1A and HIF2A in all three hPSCs lines and their differentiated progeny cultured in three different oxygen conditions were analysed with qPCR and Western blotting assays. The results of SHEF1 cells showed there was significant downregulation in the relative gene expression of HIF1A in undifferentiated cells cultured in both hypoxic conditions (2%PG and 2%WKS), and in all time course points from spontaneous differentiation cells comparison to those cultured in air oxygen (21%ST). The level of HIF1A protein was increased in day 5 from differentiating cells following treatment with 2%PG and 2% WKS, in day 20 in 2%WKS but no significant change was noticed **(Figure 4.21)**. In addition, the presence of low oxygen did not significantly impact on HIF2A gene expression in all time course points tested. In contrast, the level of HIF2A protein was increased significantly in undifferentiated SHEF1 cells cultured under 2%PG and 2%WKS (1.89 ± 0.31 and 1.92 ± 0.38, respectively) when compared with air oxygen, and in day 5 from spontaneous differentiation in 2%PG and 2%WKS (2.9 ± 0.84 and 2.65 ± 0.65, respectively) and day 10 (2.06 ± 0.14 and 2.23 ± 0.65, respectively) **(Figure 4.21)**.

Similar significant reduction was observed in gene expression of HIF1A gene expression in undifferentiated SHEF2 cells grown under 2%PG conditions (0.78 ± 0.05), and in day 5, 10 and 20 from differentiation in 2%PG (0.86 ± 0.08, 0.78 ± 0.09 and 0.67 ± 0.08, respectively) and 2%WKS (0.68 ± 0.05, 0.68 ± 0.12 and 0.67 ± 0.17, respectively) compared with the air oxygen **(Figure 4.22)**. No significant change in the protein levels of HIF1A was noticed in SHEF2 cells and their differentiated progeny in all time course points tested.

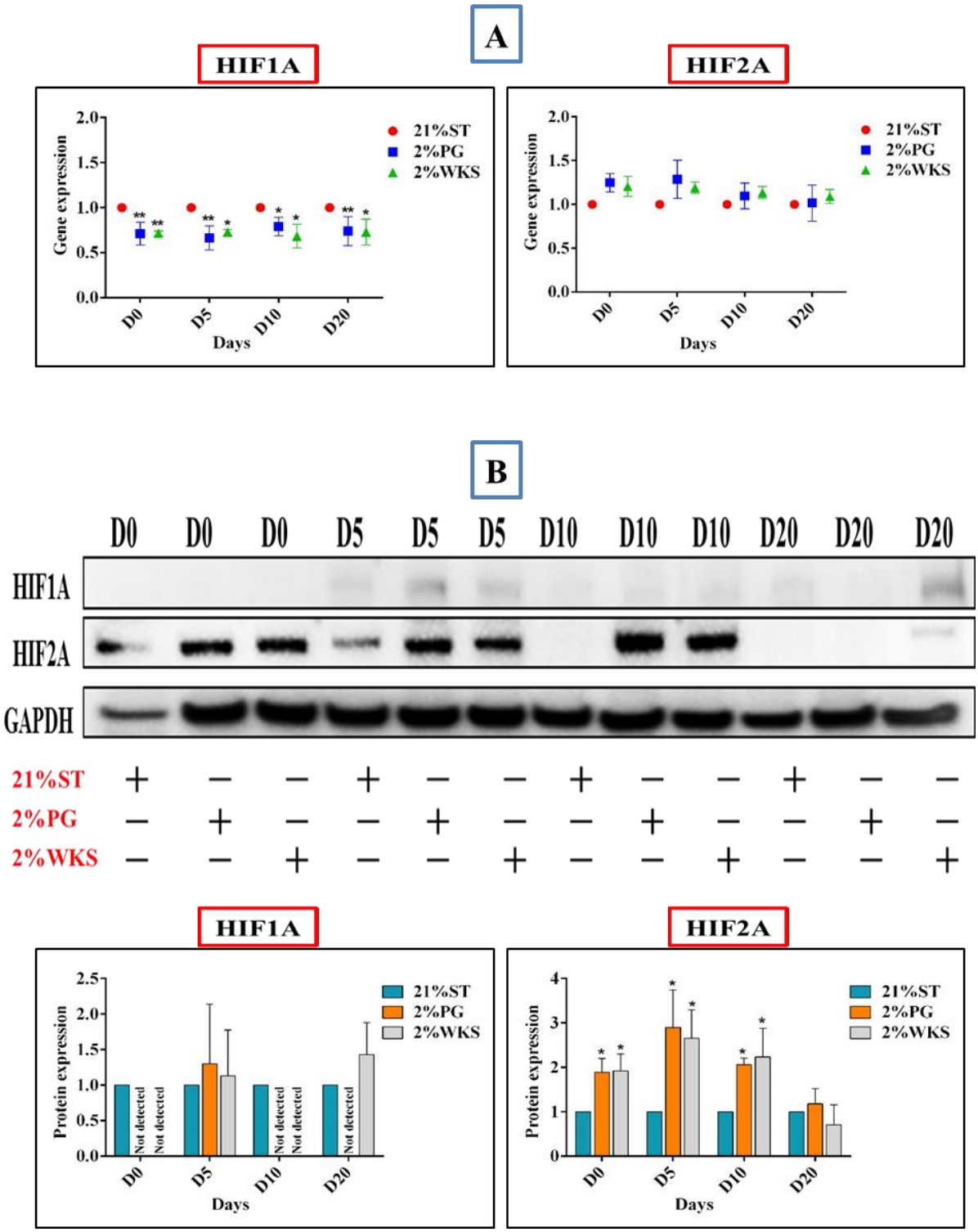


Figure 4.21 The level of HIFs expression in SHEF1 cell line and their differentiated progeny cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIF1A and HIF2A normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST). Data were

Furthermore, a significant up regulation in the HIF2A protein expression was showed under 2%PG and 2%WKS conditions in day 10 of differentiation (2.69 ± 0.65 and 1.94 ± 0.22, respectively), after 20 days (2.56 ± 0.71 and 3.39 ± 0.62, respectively) compared with the air oxygen (Figure 3.16). Also, there was a significant difference in the level of HIF2A protein between 2%PG and 2%WKS in day 5 from spontaneous differentiation (2.05 ± 0.8 and 0.5 ± 0.29, respectively). No significant change in the gene expression levels of HIF2A was noticed in SHEF2 cells and differentiated progeny **(Figure 4.22)**.

Under 2%PG and 2%WKS there was no significant changes in gene expression of HIF1A was noticed in undifferentiated hiPSC line (ZK2012L) and their differentiated progeny **(Figure 4.23)**. HIF1A protein was not detected in hiPSC line (ZK2012L) and their differentiated progeny. There was a significant increase in gene expression of HIF2A in 2%PG in undifferentiated hiPSC line (ZK2012L), and in day 5 spontaneous differentiation (1.4 ± 0.08 and 1.4 ± 0.1, respectively) compared with air oxygen. Furthermore, a significant increase in the protein level of HIF2A was showed under 2%PG and 2%WKS conditions in undifferentiated cells (1.89 ± 0.18 and 2.08 ± 0.5, respectively), and after 10 days (2.89 ± 1.0 and 2.54 ± 0.32, respectively) and day 20 in 2%PG and 2%WKS (2.47 ± 0.7 and 3.48 ± 0.7, respectively) compared with the air oxygen **(Figure 4.23)**. There was a significant difference in the level of HIF2A protein between 2%PG and 2%WKS in undifferentiated cells.

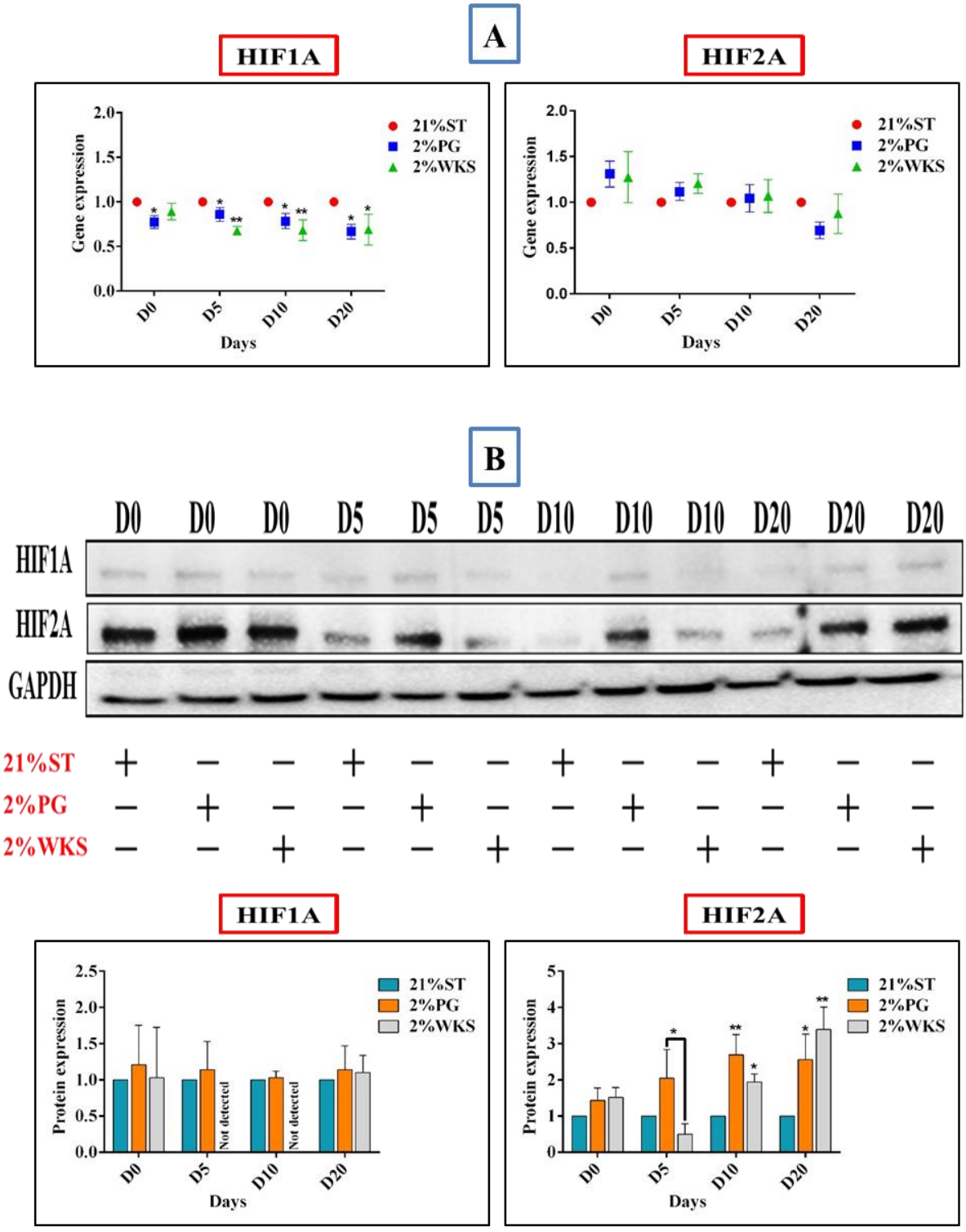


Figure 4.22 The level of HIFs expression in SHEF2 cell line and their differentiated progeny cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIF1A and HIF2A normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

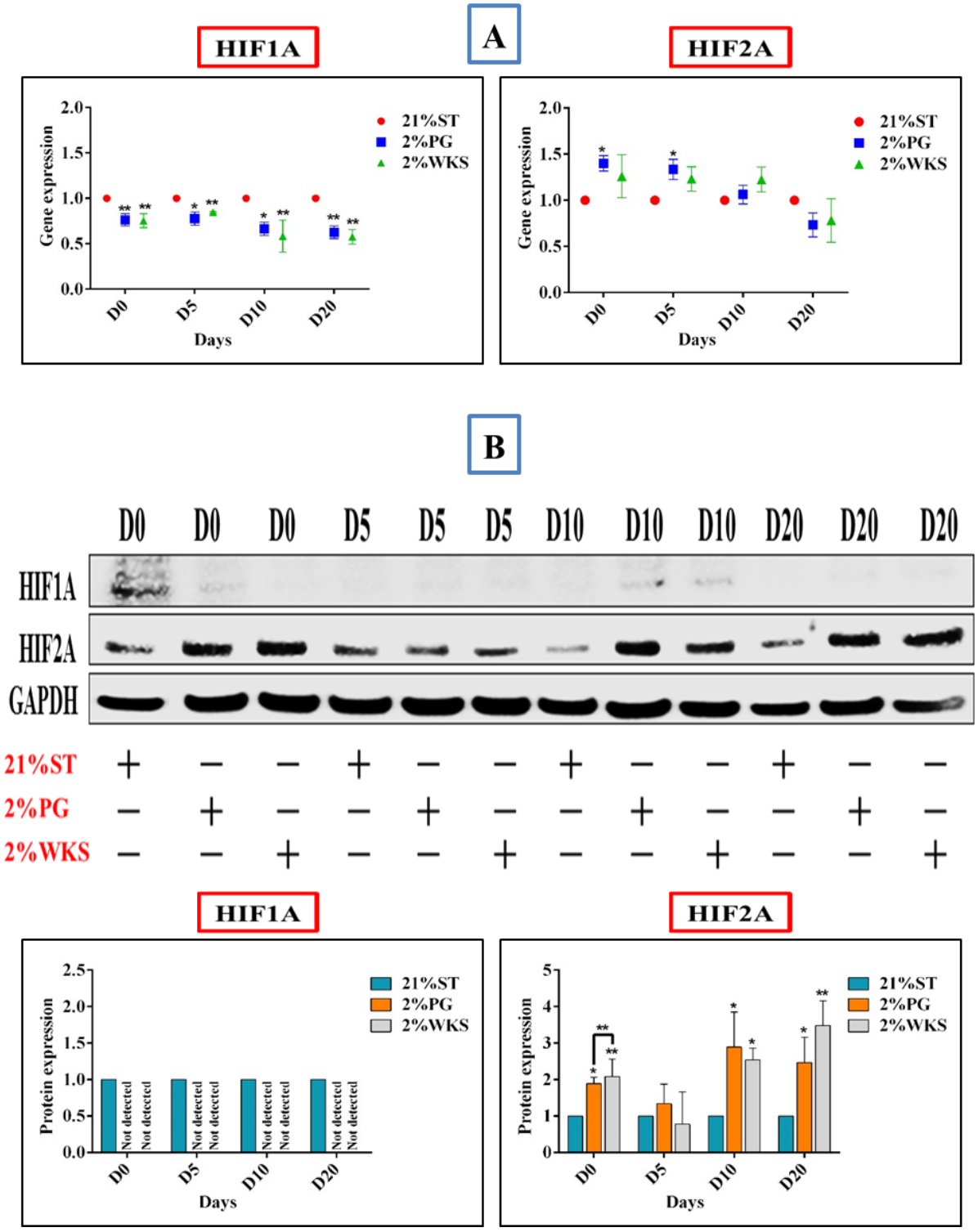


Figure 4.23 The level of HIFs expression in hiPSC line (ZK2012L) and their differentiated progeny cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIF1A and HIF2A normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

4.5 Discussion

In the last two decades the number of epigenetics studies have increased significantly into how epigenetics marks affect the characteristics and function of cells. Among these epigenetics marks is DNA methylation (5mC and 5hmC). The role of DNA methylation (5mC) in regulation of cell behaviour and gene expression in several biological processes such as cell differentiation, development and tumorigenesis are well-studied, but the role of 5-hydroxymethylated (5hmC) that has recently been detected in mammalian cells in maintenance of transcriptional regulation and differentiation are still being investigated (Chen et al, 2012). In addition, the effects of low oxygen tension on the morphology, proliferative and maintenance of the pluripotent state of human embryonic stem cells (hESCs) has been explored in many recent studies (Forsyth et al., 2006; Narva et al., 2013; Agrawal et al., 2016). In this chapter, we focussed on two parts, to test the hypothesis that the epigenetics marks (5mC and 5hmC) can be altered in response to a specific microenvironmental stressor such as reduced oxygen, and to investigate the effects of physiological normoxia on characteristics of pluripotent stem cells (hPS) cells. During all our experiments, special attention was taken to make sure that the cells were cultured under strictly controlled normoxia physiological conditions.

In this study, our results showed that hypoxic conditions (2%PG and 2%WKS) increased the proliferation rate of hPS cells as compared with air oxygen and this finding is in line with previous studies which have demonstrated that the proliferation rate of hPS cells was increased under low oxygen culture (Forristal et al., 2010; Rajala et al., 2011). In addition, our MTT and Alamar blue results displayed similar effect to growth rate **(Figure 4.2 and 4.3)**, which indicates that hPS cells have the ability to grow better under low oxygen conditions and also might refer to the balance between proliferation and metabolic activity. However, many studies revealed that hPS cells maintained under low oxygen conditions

increased the proliferation rate, improved morphology, reduced chromosomal abnormalities, increased expression of pluripotency markers, and showed less spontaneous differentiation (Forsyth et al. 2006; Forristal et al. 2010; Narva et al., 2013). Accordingly, mimicking the specialized local microenvironment or 'niches' where stem cells reside *in vivo* (the physiological normoxia level) may be beneficial for the propagation of hPS cells *in vitro* (Mazumdar et al., 2010). On the other hand, analysing proliferation rate of hES cells cultured under three different low oxygen conditions revealed no significant differences in growth rate among different hypoxic conditions compared to air oxygen level (Ezashi et al., 2005). These variations of response of hPC cell lines to reduced oxygen may be due to the variability between cell lines in addition to different metabolic requirements of these cell lines. Also, the variation between culture conditions may influence the proliferation and differentiation of many cells (Sullivan et al., 2010; Redshaw and Loughna, 2012).

Immunocytochemistry results of undifferentiated hPS cells showed that our three cell lines have typical characteristics of the surface antigen of hES and are distinct from mouse embryonic stem cells **(Figure 4.4)**. Our results are agreeing with previous reports that demonstrated that the presence of a common set of pluripotency markers such as SSEA-4(+), TRA-1-60(+) and SSEA-1(-) provides a valuable operational indication of hES cells (Adewumi et al., 2007; Tavakoli et al., 2009). To further characterize of the pluripotency status, the expression of cell surface markers was quantified by the flow cytometry. The results demonstrated that three cell lines expressed the hPS cells markers (SSEA-4 and TRA-1-60), and there was no significant difference between low oxygen and air oxygen condition **(Figure 4.5)**. However, many studies have demonstrated that cultured hPS cells under low oxygen level does not affect the expression of surface antigens SSEA-1, SSEA-4, TRA-1-60 during normal passaging (Forsyth et al, 2006; Chen et al, 2009; Forristal et al., 2010; Narva et al., 2013). In fact, many laboratories are used SSEA-4, TRA-1-60 and TRA-1-81

as a good indicator to confirm the undifferentiated state of hESCs (Adewumi et al., 2007). Recent reports revealed that silencing of core pluripotency transcription genes such as OCT-4, SOX-2 and NANOG lead to increased expression of SSEA-1 and decreased the expression of SSEA-3, TRA-1-60 and SSEA-4 markers as well as to induce differentiation and rapid loss of pluripotency of hPS cells (Matin et al., 2004; Fong et al, 2008; Narva et al., 2013).

Routine culture of undifferentiated hPS cells in hypoxic conditions resulted in a significant increase in expression of OCT-4, SOX-2 and HIF-2 at transcriptional and protein level. There are several pathways and transcription factors involved in the maintenance of hESCs self-renewal, including OCT-4, NANOG, and SOX-2. These regulate self-renewal of hESCs by activating or repressing patterns of gene expression mediated by other transcription factors, but the molecular mechanisms underlying modulation of self-renewal and differentiation of hPS cells under low oxygen conditions remains unclear (Boyer et al. 2006; Pan et al. 2002). However, *in vitro* studies by Mazumder *et al* using reduced oxygen culture conditions (< 5% O_2) revealed activation of Wnt signalling through HIF1A by enhancing β‑catenin activation which in turn stimulated target genes. These studies observed that the growth rate of physiological normoxia ES cells was increased when compared with normoxic cells under induction of Wnt signalling condition (Mazumder et al., 2010). Similarly, Barbosa et al, using reduced oxygen mES cells culture revealed a significant role of GSK-3- mediated signalling in stimulation of the Wnt/β‑catenin pathway in regulation of the core of cell pluripotency transcription (e.g. OCT-4) as well as maintenance of mESCs self-renewal (Barbosa et al., 2012). The effect of HIFs in ESCs through Wnt/β‑catenin pathway was confirmed in another recent study (Sun et al., 2015). In addition, Silencing of HIF2A or HIF3A in low oxygen, leads to the decrease of OCT-4, SOX-2 and NANOG transcription factors (Forristal et al., 2010). On the other hand, silencing of core pluripotency

transcription factors OCT-4, NANOG and SOX-2 leads to induce differentiation and rapid loss of pluripotency (Matin et al., 2004; Fong et al., 2008).

In the second part of this study, we examined the role of reduced oxygen in alteration of the global DNA methylation content (5-mC and 5-hmC) in hESCs and their differentiated cells. This was achieved with an ELISA-based colorimetric assay to estimate DNA methylation content directly and was complemented by determining the expression of DNA methyltransferase (DNMTs) and ten-eleven translocation methylcytosine dioxygenase (TETs) enzymes via RT-PCR assay and Western bloting.

The results of a one-way ANOVA showed a significant effect of hypoxia on the global 5mC and 5hmC levels of hPS cells and their spontaneous differentiation **(Section 4.4.6)**. These results suggested that physiological O_2 concentrations induce changes in global DNA methylation and are consistent with previous evidence that indicated that the changes in both DNA methylation and hydroxymethylation play an important role in PSCs functions under physiological normoxia conditions (Waston et al., 2010; Mariani et al., 2014). However, two recent studies have demonstrated that global levels of both 5mC and 5hmC significantly decreased when hES cells underwent differentiation (Kim et al., 2014; Sun et al., 2014). Results by Mariani et al., showed that levels of 5hmC increased after treatment with 1% O_2 (hypoxia) in tumorigenic cell lines but not in nontumorigenic cells. Furthermore, quantitation of the global levels of 5mC and 5hmC by HPLC-MS/MS in SK-N-BE cells (neuroblastoma) exposed to 24, 48, or 72 hours of low oxygen demonstrated that global levels of 5hmC increased at 48 and 72 hours in reduced oxygen, whereas the global levels of 5mC remained unchanged at all timepoints (Mariani et al., 2014). In addition, cells maintained at lower oxygen levels (1%) revealed decreases in the global levels of DNA methylation, whereas in the absence of HIF1A, the global level of DNA methylation increased accompanied by an increase in DNA methyltransferase (DNMT3B) expression

(Waston et al., 2009; Waston et al., 2010). Changes in DNA methylation can impact the control of HIFs binding to target genes directly by methylation of a specific CpG dinucleotides within the hypoxia response elements (HRE) which are present in promoters, introns, and 3´ enhancers (Rossler et al., 2004; Waston et al., 2010). In addition, changes in DNA methylation are crucial for stem cell differentiation as methylated DNA alters the interaction between transcription factors and their binding sites (TFBSs) on DNA (Chen et al., 2011). Furthermore, Altun *et al* demonstrated that key transcription factors of embryonic stem cells which are involved in maintaining the ESCs in undifferentiated state are unmethylated in ESCs but methylated in differentiated cells (Altun et al., 2010).

To test the hypothesis that changes of global DNA methylation (5mC and 5hmC) in response to reduced oxygen could be related to changes in expression of DNMTs/TETs, the expression of DNMTs and TETs enzymes at the mRNA and protein level were performed at undifferentiated and differentiated cells exposed to different oxygen conditions in four different time points (0, 5, 10 and 20 days). Our results showed a significant decrease in the expression of de novo methylation DNA methyltransferase DNMT3B and hydroxylases 5mC DNA TET1 in response to low oxygen at transcription and protein level. Notably, this may correlate to the decrease in global 5mC and 5hmC level observed in hPS cells cultured in 2%PG and 2%WKS **(Sections 4.4.7 and 4.4.8)**. In contrast, the expression of DNMT3B at promoter regions increased significantly under reduced oxygen in compared to air oxygen condition **(Section 4.4.9)**. However, it has been reported that down-regulation of DNMTs under low oxygen were associated with global DNA hypomethylation in human colorectal cancer cell lines (Skowronski et al., 2010). Our results also agree with previous studies that showed that low oxygen upregulated the expression of DNMT1 and DNMT3A but not DNMT3B and induced genomic DNA hypomethylation in Hepatoma cells (Liu et al., 2011). In contrast, increased expression of DNMT1 and DNMT3D was associated with induced

global DNA hypermethylation when human cardiac fibroblast cells cultured in 1% hypoxia (Waston et al., 2014). In addition, the recent study showed that level of TET1 have tendency to decrease in normal fibroblasts cells (FB) cultured under low oxygen condition (Hattori et al., 2015). Furthermore, Thienpont et al demonstrated that level of TET1 which was down-regulated in mESC cultured under low oxygen (physiological O_2 concentration) led to decrease in the level of 5hmC (Thienpont et al., 2016). However not much is known about the mechanism of effect of reduced oxygen and the changes in methylation status.

In conclusion, understanding the mechanisms involved in pluripotent epigenetic in vitro is essential for further understanding of the development of functional cells, with the potential for clinical translation into areas such as prognosis and treatment (Baylin and Jones, 2011). This study has highlighted the significant effect a hypoxic microenvironment can have on epigenetic changes within hPS cells and their differentiation progeny, and the downstream effects it can have on the physiological behaviour of cells including proliferation and metabolic activity.

Chapter 5: Tumour hypoxia reduces DNMT3B activity with accompanied DNA hypomethylation in human cancer cells

5.1 Introduction

In the previous chapters, the results have highlighted the potential role of low oxygen tension in the induction of DNA hypomethylation in both pluripotent and multipotent stem cells. This chapter investigated the role of reduced oxygen in the modification of DNA methylation marks (5mC and 5hmC) in cancer cells. Despite improvements in diagnosis and treatment of cancer, it is still one of the major causes of death in the world alongside heart disease (WHO, 2015). Cancer is a disease characterized by uncontrolled cellular proliferation and differentiation (Parkin et al., 1999; Alghamdi and Khorshid, 2012). It is known to affect different organs or tissues in the body such as liver, lung, breast and ovaries etc. Traditionally cancer has been viewed as a disease characterised by the accumulation of genetic mutations, however extensive research into cancer epigenetics over the past decade has come to challenge this view (Hanahan and Weinberg, 2011; You and Jones, 2012).

Several findings have shown that cancer appears to have both an epigenetic and genetic basis (Sadikovic et al., 2008; Baylin and Jones, 2012). Recent studies have revealed that epigenetic mechanisms are a main player in cancer-associated disorders and that the accumulation of epigenetic modifications could be a mechanism underlying these disorders (Sharma et al., 2010; Wong et al., 2017). Dysregulation of the epigenetic machinery and subsequently the maintenance of epigenetic marks can result in inappropriate activation and/or inhibition of gene expression, contributing too many diseases, most notably cancer (Sharma et al., 2010). Among epigenetic alterations DNA methylation is that the most widely studied in mammals due to the increased availability of commercial genomics assays that are well-matched with different tissue samples (Kurdyukov and Bullock, 2016; Massie et al., 2017). DNA methylation patterns are established through the addition of a methyl group at the 5th carbon position of cytosine residues by DNA methyltransferases (DNMTs), to form 5-methylcytosine (5mC). Currently, three ten-eleven translocations 1–3 (TET1–3) proteins

have been identified in many mammals. TET proteins are recently known have the ability to catalyze the conversion of 5mC into 5hmC, with distinct regulatory function (Kanwal and Gupta, 2012; Putiri et al., 2014; Green et al., 2016).

In cancer, hypermethylation of promoter CpG islands of tumour suppressor genes (TSG) is one of the most common mechanisms leading to silence these genes during tumorigenesis (Sadikovic et al., 2008; Subramaniam et al., 2014). In contrast to genetic mutation cancer causes, the epigenetic codes in cancer are potentially reversible. For instance, DNA hypermethylation can be reversed by inhibition of DNMTs putatively leading to the reactivation of expression of tumour suppressor genes. This presents a potentially effective approach to the development of new therapeutic targets for cancer treatment (Fahy et al., 2012; Yu et al., 2016). Subramaniam *et al* reported that inhibition of DNMT expression led to a decrease in tumour formation through the re-activation of the tumour suppressor genes (Subramaniam et al., 2014). On the other hand, it is established that the development and progression of a large number of cancers are influenced by the interactions between cancer cells and their local tumour microenvironment (Hockel and Vaupel, 2001). The oxygen tension within cancer tissues can range from physiological levels of ~2-7% to severely hypoxic tensions of <1% (Heddleston et al., 2010; Thirlwell et al., 2011). Despite this, cancer cells often survive and thrive within these conditions by several critical alterations that allow tumour cell survival, such as apoptosis inhibition, an angiogenic phenotype, and altered glucose metabolism (Fruehauf and Meyskens, 2007). However, it has been hypothesized that hypoxic microenvironments may influence alterations in DNA methylation, resulting in inappropriate silencing or activation of genes involved in the progression of cancer (Shahrzad et al., 2007). Watson *et al* study revealed that cells maintained in severe oxygen tension (1%) displayed reduced global DNA methylation (Waston et al., 2009). Similarly, a decrease in 5mC levels was observed in *in vivo* xenograft cancer cells following exposure to

severe hypoxic conditions (Shahrzad et al., 2007). In addition, it was reported that HIF1A regulated DNMT1 and DNMT3B expression in cardiac tissue fibrosis resulting in altered the DNA methylation level (Waston et al., 2014). In addition, there is increased evidence that hypoxia can induce changes in global deacetylation and alterations in acetylation and histone methylation in promoter regions that are related to hypoxia genes (Thirlwell et al., 2011). Consequently, these studies highlight the significant correlation between hypoxia and the modification of DNA methylation in cancer. However, the DNA methylation pattern has been widely studied in cancer cells but the role of hypoxia in regulation of the methylation level and in particularly in regulation of the 5hmC level and TETs enzymes regulation is still under investigation (Chen et al., 2012).

5.2 Amis

In this chapter we aim to determine the role of hypoxia in the regulation of epigenetic marks in cancer cells. To examine this, we aim to:

1. Investigate the effects of hypoxia on the proliferation, expression of core pluripotency factors and HIFs in cancer cells.

2. Investigate the effects of hypoxia on global DNA methylation (5mC and 5hmC) in cancer cells.

3. Assess the relationship between changes in global DNA methylation (5mC and 5hmC) patterns in response to oxygen modulation and the expression of DNMTs/TETs.

4. Investigate the effects of hypoxia on the local DNA methylation patterns at selected CpG sites within the promoter regions of DNMTs/TETs genes in cancer cells

5.3 Methods

5.3.1 Human cancer cell lines

All cancer cell lines used in this study were obtained from the laboratory stock of Guy Hilton Research Centre, Keele University as described in **Table 5.1**. The cells were incubated at 37ºC at three different oxygen tensions as described in **Section 2.2.1** and detached and passaged as described in **Section 2.2.3**. To maintain the oxygen level of the cells during physiological oxygen culture, cell culture media was deoxygenated to predefined oxygen concentration (2% O_2) as described in **Section 2.2.2**.

Table 5.1 Human cell lines used in this chapter

Cell line	Tumor type	Reference
A549	Lung carcinoma	Giard et al., (1973)
COV362	Ovarian cancer	Berg-Bakker et al., (1993)
MG-63	Osteosarcoma	Billiau, (1975)
SH-SY5Y	Neuroblastoma	Biedler et al., (1978)
Jurkat	T cell leukemia	Schneider et al., (1977)
IMR-90	Normal lung fibroblast	ATCC® CCL-186™

5.3.2 Measurement of cellular viability

Changes in cellular viability following hypoxia treatment were assessed as outlined in **Chapter 2**, **Section 2.6.1** and **2.6.2**.

5.3.3 Global 5mC and 5hmC analysis

Total genomic DNA was isolated from the cancer cells at different time points as described in **Section 2.7.2**. DNA, 100ng and 200ng, was subjected to methylated DNA (5mC) and hydroxymethylated DNA (5hmC) quantification assay (Colorimetric), respectively, with the MethylFlash quantification kit as outlined in **Section 2.7.3**.

5.3.4 Quantitative real-time RT-PCR

Total RNA was extracted from the cancer cells and their differentiated progeny at different time points as described in **Section 2.8.1**. Relative gene expression was carried out in triplicate samples by using the QuantiFast SYBR Green OneStep RT-PCR kit **(Section 2.8.3)**.

5.3.5 Protein analysis

Cancer cells were cultured on 6 well-plate and cells lysed as described in **Section 2.9.1** before 30 µg of total protein **(Section 2.9.2)** was subjected to western blot analysis using antibodies against DNMT3B, TET1, HIF1A and HIF2A as described in **Chapter 2**, **Section 2.9.4**.

5.3.6 Pyrosequencing of sodium bisulphite-converted DNA

500ng of genomic DNA was subjected to bisulphite conversion before pyrosequencing was achieved using a PyroMark Gold Q24 Reagents and PyroMark Q24 Software 2.0 (Full details in **Chapter 2**, **Section 2.10**).

5.4 Results

5.4.1 Effect of reduced oxygen on the proliferation of cancer cells

Proliferation of the human cancer cells cultured in air oxygen (21%ST) and reduced oxygen conditions (2%PG and 2%WKS) was determined using a cell counting assay. The number of cells seeded into multiple wells of a 6-well plate were counted daily using a counting chamber for 6 days. Four cell lines (A549, COV362, MG-63 and Jurkat cells) showed a significant decrease in the proliferation rate of cells cultured under reduced oxygen conditions (2%PG and 2%WKS) in comparison to those cultured in air oxygen ($p<0.05$ or

$p<0.01$) **(Figure 5.1)**. Also, A549 and Jurkat cells cultured under 2%WKS condition showed the lowest proliferation rate ($p<0.01$) in comparison to cells cultured in 2%PG and air oxygen at day 6 of the experiment. In contrast, the results showed there was increase in the proliferation rate of SH-SY5Y cells cultured under 2%WKS conditions when compared to those in 2%PG and 21%ST. In addition, the results of non-cancer cell line (IMR-90) showed there was no difference in the proliferation rate between cells cultured under reduced oxygen conditions or air oxygen **(Figure 5.1)**.

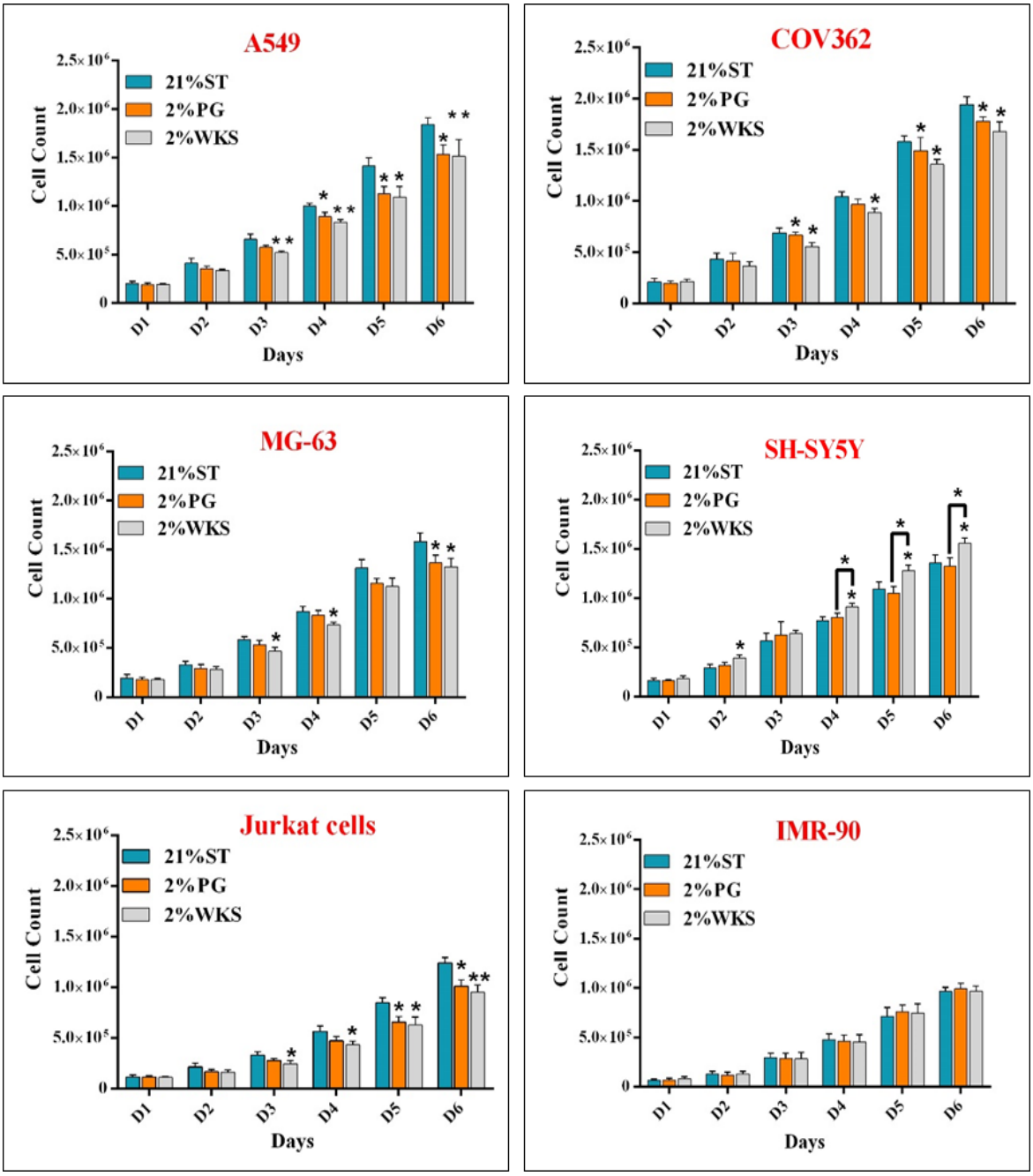


Figure 5.1 Effect of reduced oxygen on the proliferation rate of cancer cells. Cell count experiments were conducted over 6 days after incubation in air oxygen (21%ST) and physiological normoxia conditions (2%PG and 2%WKS), with media changed every day. Y-axis indicates cell number x 10^6. X-axis indicates time (days). Data are shown as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.2 Reduced oxygen decreases metabolic activity in cancer cells

MTT assay was used to analyse the metabolic activity of cancer cells cultured in air oxygen (21%ST) and reduced oxygen conditions (2%PG and 2%WKS). Similar to above a decrease in MTT was noticed for cancer cells cultured in reduced oxygen conditions in comparison to those cultured in air oxygen **(Figure 5.2)**. A549 displayed a significant decrease in MTT at days 2 ($p<0.01$), 4, 5 and 6 in cells cultured under 2%PG condition ($p<0.05$), and at day 5 and 6 in 2%WKS conditions ($p<0.01$ and $p<0.05$, respectively) in comparison to cells cultured in air oxygen (21%ST) **(Figure 5.2)**. COV362 cells cultured in 2%PG and 2%WKS had a lower proliferation potential than those cultured in air oxygen ($p<0.05$), whereas MG-63 exhibited significant MTT decreases in both reduced oxygen conditions ($p<0.05$) at day 4, 5 and 6 in comparison to cells cultured in air oxygen **(Figure 5.2)**. The results also showed that the cell viability of Jurkat cells was decreased significantly ($p<0.05$) in 2%WKS at days 3 and thereafter, and at days 3 and 6 ($p<0.05$) in cells cultured in 2%PG in comparison to cells cultured in air oxygen. In contrast, the results showed the metabolic activity increased significantly ($p<0.05$) of SH-SY5Y cells cultured under 2%WKS conditions at days 3 and thereafter in comparison to cells cultured in air oxygen. Also, the results of non-cancer cell line (IMR-90) showed there was no difference in the metabolic activity between cells cultured under reduced oxygen conditions or air oxygen except the cells cultured in 2%WKS at days 5 and 6 ($p<0.01$ and $p<0.05$, respectively) in comparison to cells cultured in air oxygen **(Figure 5.2)**.

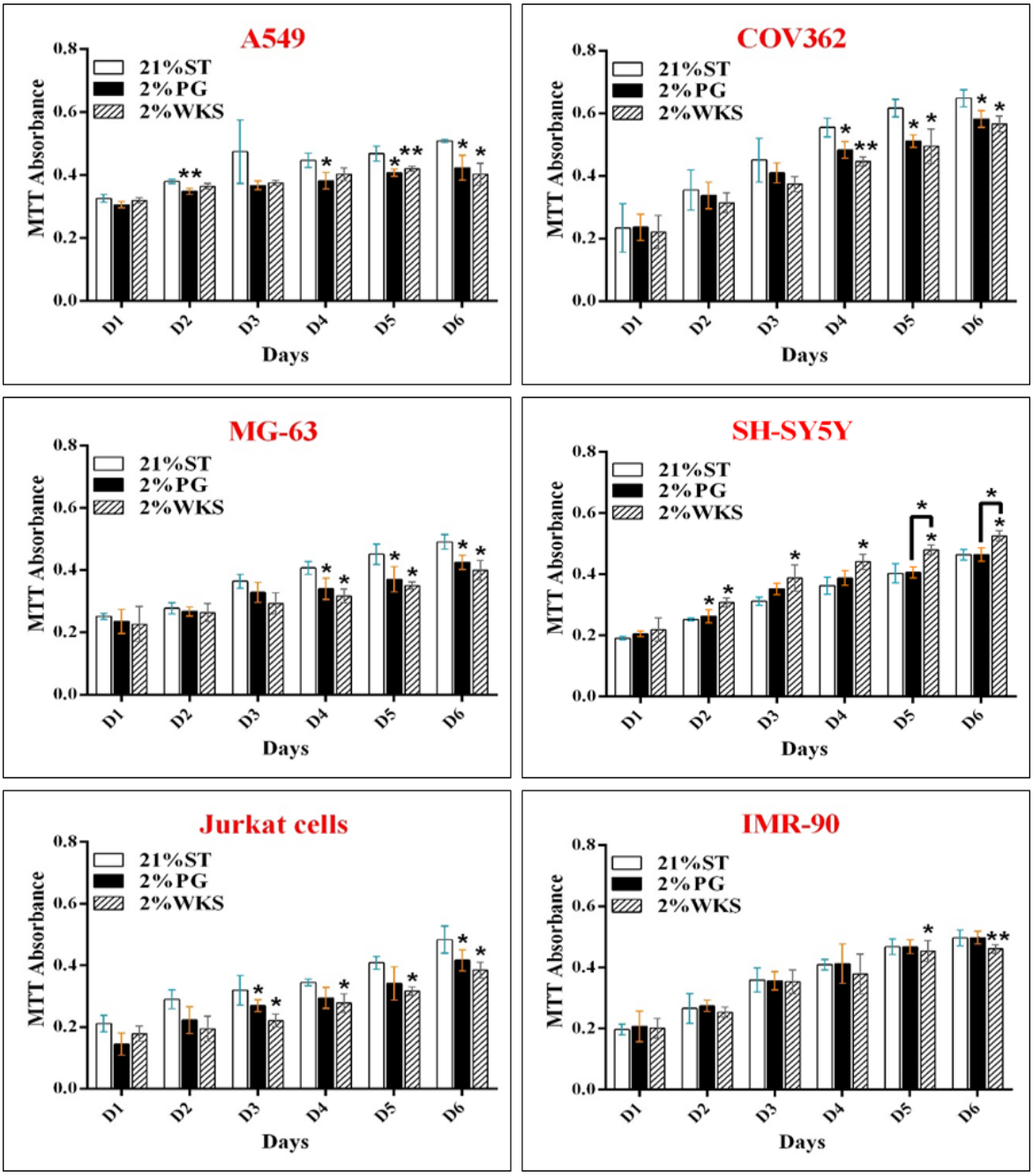


Figure 5.2 Effect of reduced oxygen on the viability of cancer cells. MTT absorbance (570 nm) was measured over 6 consecutive days in cancer cells incubated under air oxygen (21%ST) and physiological normoxia conditions (2%PG and 2%WKS). Y-axis indicates MTT absorbance (570 nm). X-axis indicates time (days). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.3 Hypoxia induces stem cell markers in cancer cell lines

Stem cells are localized in a specialized microenvironment characterized by their low oxygen levels, which can regulate the balance among self-renewal, differentiation, and stem cell quiescence. In tumors, hypoxia is associated with aggressive disease course and poor clinical outcomes (Mohyeldin et al., 2010). In addition, several cancers have been shown to display gene expression signatures characteristic of human embryonic stem cells (hESC) (Mathieu et al., 2011). However, it is not well understood how cancer cells respond to reduced oxygen availability. Therefore, we analyzed the effect of low oxygen concentrations (2%PG and 2%WKS) on the expression of transcription factors OCT-4 (POU5F1), NANOG and SOX-2 in cancer cells. Reduced oxygen led to enhanced mRNA levels of pluripotency markers; OCT-4 and NANOG but not SOX-2 **(Figure 5.3)**. However, the relative fold expression of OCT-4 was significantly increased in 2%WKS cultures compared to air oxygen 21%ST in A549, COV362, SH-SY5Y and Jurkat cells (2.24 ± 0.46, 1.8 ± 0.34, 1.7 ± 0.24 and 1.5 ± 0.18, respectively), and in 2%PG cultures compared to air oxygen 21%ST in A549 (2.1 ± 0.62) **(Figure 5.3)**. NANOG expression was generally higher in low oxygen than air oxygen but was not significantly upregulated except in the non-cancer cell line (IMR-90) cultured in 2%PG and 2%WKS (1.4 ± 0.23 and 1.4 ± 0.22, respectively) compared to air oxygen 21%ST. In contrast, SOX-2 expression was generally lower in reduced oxygen than air oxygen but was not significantly down-regulated except in MG-63 cells cultured in 2%PG (0.43 ± 0.21) compared to air oxygen as shown in **Figure 5.3**.

We next investigated the effects of removed reduced oxygen stress at the transcriptional level. First, A594, COV362 and MG-63 cells were cultured in low oxygen condition (2%WKS) for five passages before the cells were returned to air oxygen condition and cultured for five passages again {2%WKS – air oxygen (21%ST)}. Our results showed that the relative fold expression of OCT-4 was significantly decreased in 2%WKS-21%ST

conditions (1.1 ± 0.31 and 0.94 ± 0.05, respectively) compared to 2%WKS cultures in A549, COV362 cells (2.24 ± 0.46, 1.8 ± 0.34) respectively. No significant change was observed in expression of NANOG and SOX-2 in cells that cultured in 2%WKS-21%ST conditions compared to other cultures conditions **(Figure 5.3)**.

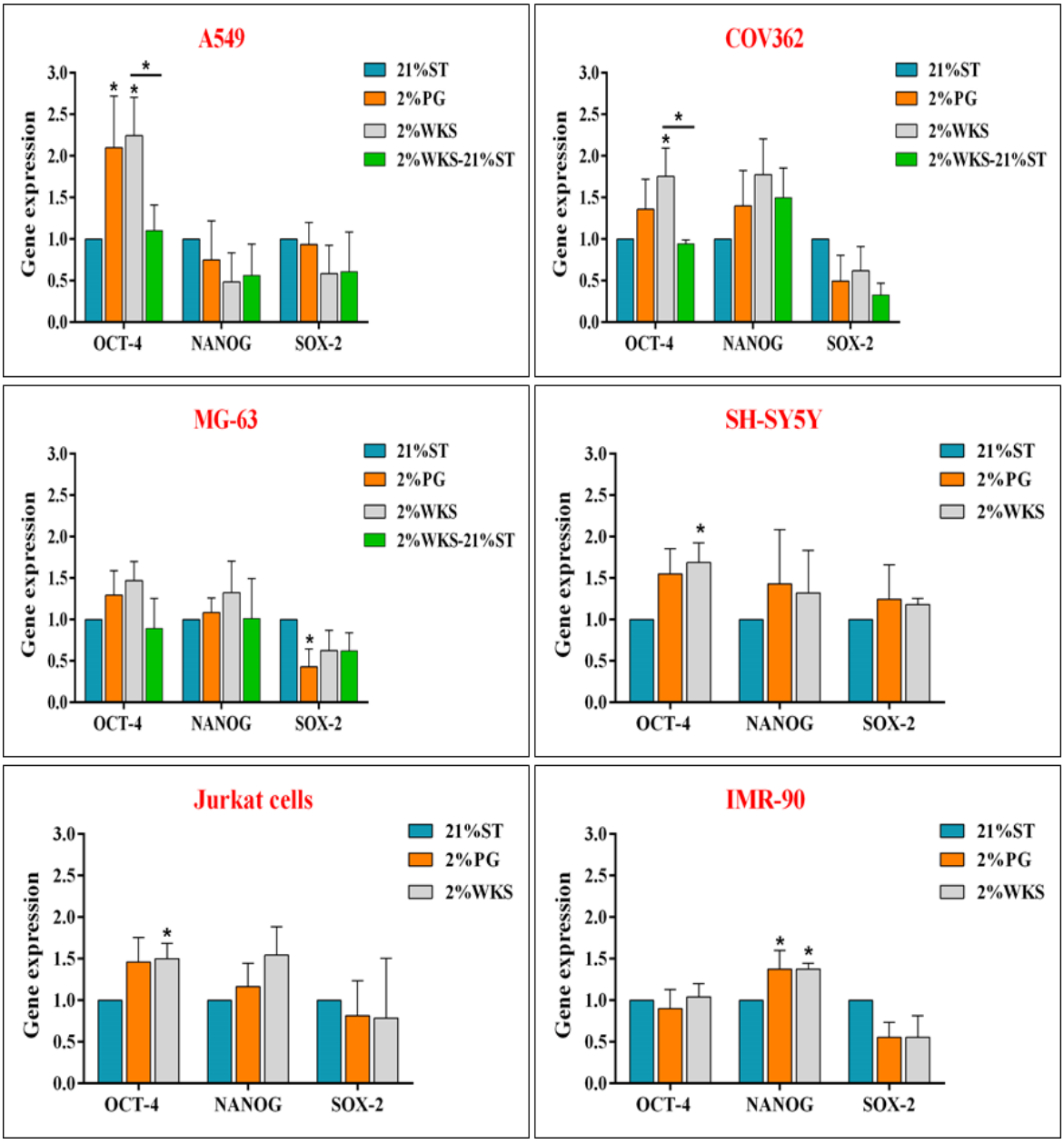


Figure 5.3 Quantitative RT-PCR analysis of OCT-4 (POU5F1), NANOG, and SOX-2 in response to different oxygen conditions in cancer cells. The expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{\wedge\Delta\Delta Ct}$ of treated cell to

untreated cell. X-axis indicates pluripotency markers. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05 vs air oxygen (21%ST).

5.4.4 Analysis of global 5mC and 5hmC analysis

5.4.4.1 Global 5- methylcytosine was decreased in cancer cells subjected to hypoxia

Global DNA methylation (5mC) was explored by an immunospecific method in human cancer cell lines after incubation in different oxygen tensions. The level of 5mC in A549 cells decreased significantly following incubation in 2%WKS (0.65 ± 0.05, $p<0.05$) in comparison to those cultured in 21%ST (0.91 ± 0.16) **(Figure 5.4)**. Also, as shown in **Figure 5.4** DNA methylation decreased significantly in COV362 cells grown in 2%PG (0.61 ± 0.08, $p<0.05$) and in 2%WKS (0.59 ± 0.051, $p<0.05$) versus 21%ST (0.8 ± 0.09). In addition, MG-63 cells cultured in 2%PG and 2%WKS showed significant reductions in 5mC content (0.67 ± 0.12 and 0.69 ± 0.06, $p<0.05$) respectively, in comparison to those cultured 21%ST (0.98 ± 0.14) **(Figure 5.4)**. SH-SY5Y cells cultured in 2%PG and 2%WKS displayed similar reductions in 5mC level (1.3 ± 0.12 and 1.2 ± 0.05, $p<0.05$) respectively, in comparison to those cultured 21%ST (1.6 ± 0.12). In contrast, no significant change in DNA methylation level was observed in Jurkat cells and IMR-90 cells that cultured in hypoxia conditions compared to air oxygen culture condition **(Figure 5.4)**.

On the other hand, a significant increase was observed in 5mC levels in A549 cells that were cultured in the 2%WKS-21%ST condition approach (1.1 ± 0.03) in comparison to those cultured in 2%PG and 2%WKS conditions alone (0.65 ± 0.05 and 0.76 ± 0.09, $p<0.05$ and $p<0.01$), respectively **(Figure 5.4)**. Similar significant difference was also noticed in COV362 cells cultured in 2%WKS-21%ST condition (0.89 ± 0.05, $p<0.01$) in comparison to those cultured in 2%PG and 2%WKS conditions. No significant change in DNA

methylation level was observed in MG-63 cells that returned to 2%WKS-21%ST condition compared to other oxygen culture conditions **(Figure 5.4).**

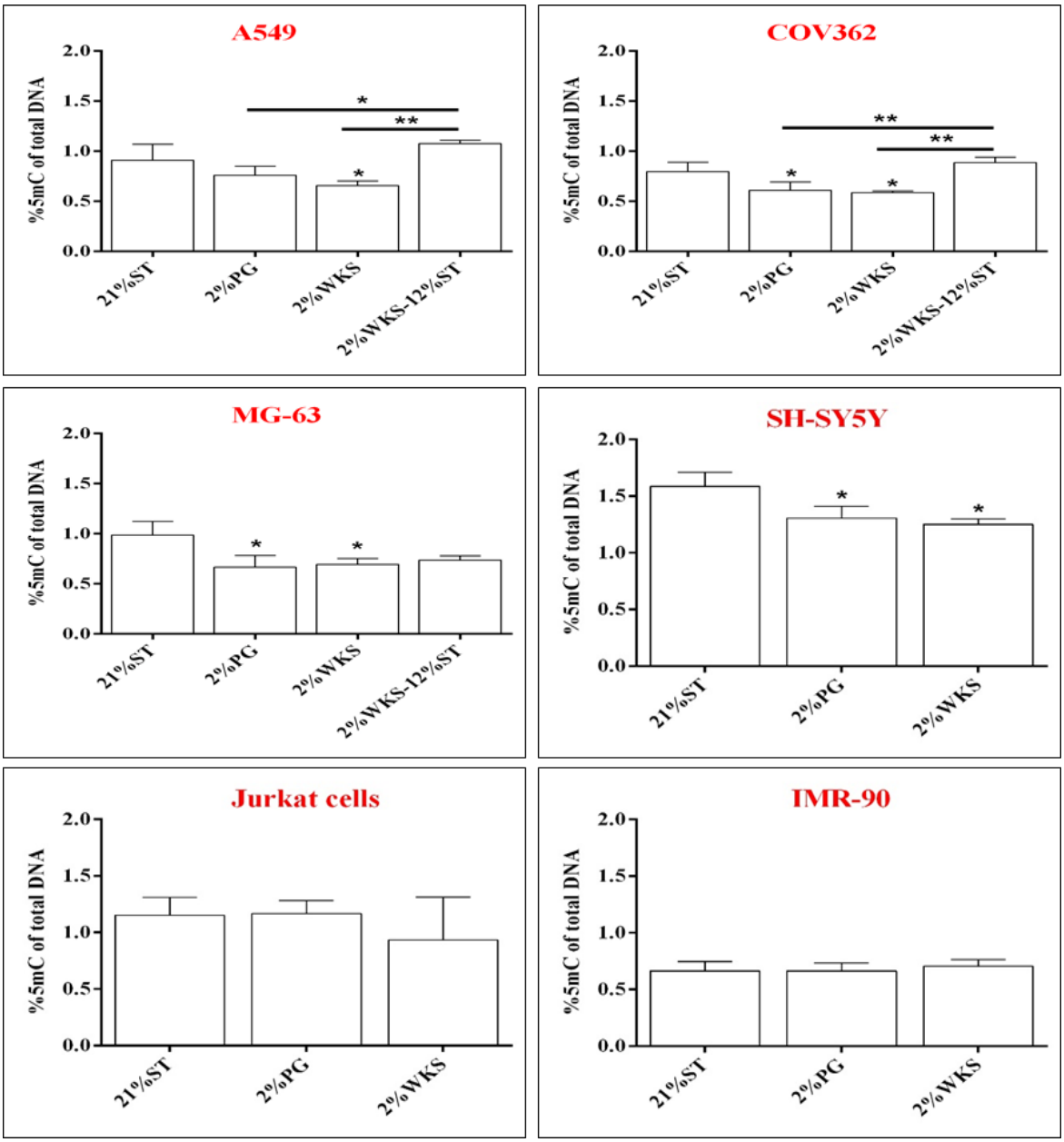


Figure 5.4 Global changes of 5-methylcytosine contents in human cancer cell lines. A set of cancer cell lines were incubated in different oxygen tensions. The methylated DNA fraction in the total isolated DNA was measured using MethylFlashTM Methylated DNA quantification kit. Y-axis indicates 5-methylcytosine absorbance (450 nm). X-axis indicates different oxygen culture conditions. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.4.2 Hypoxia induced DNA hydroxymethylation (5hmC) in cancer cells

Next, we assessed the effect of varying oxygen concentrations on the levels of DNA hydroxymethylation (5hmC) in cancer cells. We observed a significant increase in global DNA hydroxymethylation (5hmC) in A549 cells following incubation in 2%PG and 2%WKS (0.081 ± 0.026 and 0.11 ± 0.004, $p<0.05$, $p<0.01$) respectively, in comparison to those cultured in 21%ST (0.04 ± 0.015) **(Figure 5.5)**. Also, DNA hydroxymethylation increased significantly in COV362 cells grown in 2%WKS (0.08 ± 0.011, $p<0.05$) versus 21%ST (0.05 ± 0.02). In addition, SH-SY5Y cells cultured in 2%WKS showed significant increase in 5hmC content (0.14 ± 0.008, $p<0.05$) in comparison to those cultured 21%ST (0.11 ± 0.009) **(Figure 5.5)**. In contrast, no significant change in DNA hydroxymethylation level was observed in MG-63, Jurkat cells and IMR-90 cells that cultured in hypoxia conditions compared to air oxygen culture condition **(Figure 5.5)**. In addition, a non – significant decrease was observed in A549, COV362 and MG-63 cells that cultured in 2%WKS-21%ST condition compared to other oxygen culture conditions **(Figure 5.5)**.

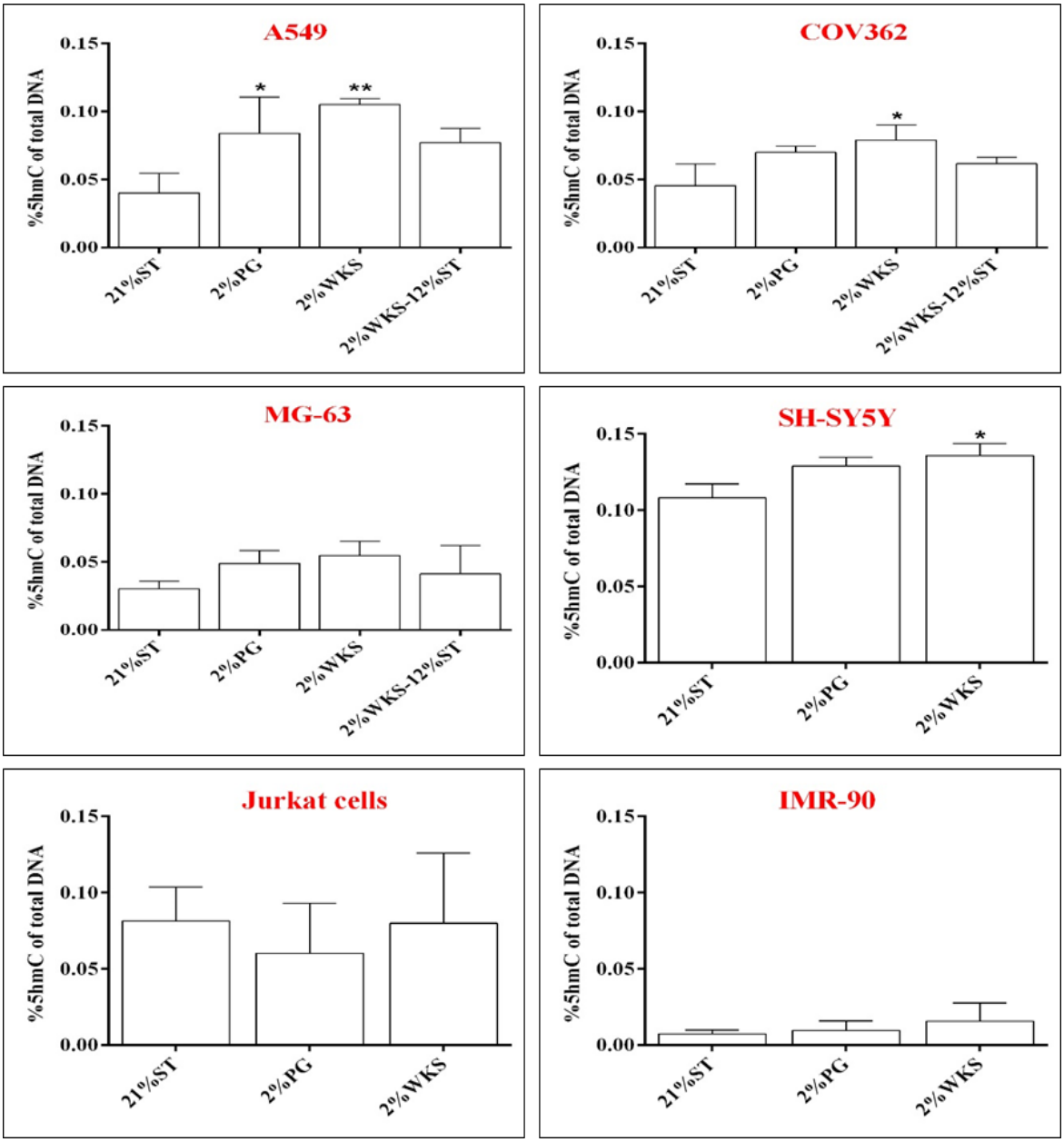


Figure 5.5 Global changes of 5-hydroxymethylation contents in human cancer cell lines. A set of cancer cell lines were incubated in different oxygen tensions. The methylated DNA fraction in the total isolated DNA was measured using MethylFlashTM Methylated DNA quantification kit. Y-axis indicates 5-hydroxymethylation absorbance (450 nm). X-axis indicates different oxygen culture conditions. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.5 Expression of DNMTs and TETs by qPCR in tumour hypoxia

5.4.5.1 Hypoxia decreases the expression of DNMT3B enzymes in cancer cells

RT-qPCR was employed to examine whether the reduction in global DNA methylation (5mC) in response to hypoxia were in relation to changes in the expression of DNMT genes. The level of DNMT3B was decreased significantly in A549 cells following treatment with 2%PG and 2%WKS (0.59 and 0.49, $P<0.05$) respectively, and in COV362 cultured in 2%PG (0.47, $P<0.05$) versus 21%ST **(Figure 5.6)**. Similar significant reductions were observed in DNMT3B level in MG-63 cells grown under 2%PG, 2%WKS and 2%WKS-21%ST conditions (0.38, 0.18 and 0.33, $P<0.01$), respectively versus 21%ST **(Figure 5.6)**. Also, significant differences were showed in expression of DNMT3B in SH-SY5Y cells cultured in 2%PG and 2%WKS (0.51 and 0.45, $P<0.01$) respectively, versus 21%ST. In addition, the level of DNMT3L was decreased significantly in SH-SY5Y cells cultured in 2%PG and 2%WKS (0.47 and 0.36, $P<0.01$) versus 21%ST **(Figure 5.6)**. IMR-90 cells displayed a significant increase in DNMT3B in cells cultured 2%WKS (0.63, $P<0.05$) versus 21%ST. In addition, the level of DNMT3A was increased significantly in MG-63 and Jurkat cells cultured in 2%WKS (0.46 and 0.70, $P<0.05$) respectively, versus 21%ST **(Figure 5.6).** No significant change was observed in DNMT3B expression in Jurkat and IMR-90 cells cultured in both hypoxia conditions in compared to air oxygen conditions.

On the other hand, a significant difference was observed in DNMT3B in A549 cells cultured in 2%WKS-21%ST condition (1.3, $P<0.01$) in comparison to those cultured in 2%PG and 2%WKS conditions (0.59 and 0.49, respectively) **(Figure 5.6)**. Similar significant difference was also noticed in COV362 and MG-63 cells cultured in 2%WKS-21%ST condition (1.3 and 1.6, $p<0.01$) in comparison to those cultured in 2%PG and 2%WKS conditions. In

addition, significant change in DNMT3L level was observed between 2%WKS-21%ST and 2%WKS (1.2 and 0.67, $p<0.05$) in A549 cells **(Figure 5.6)**.

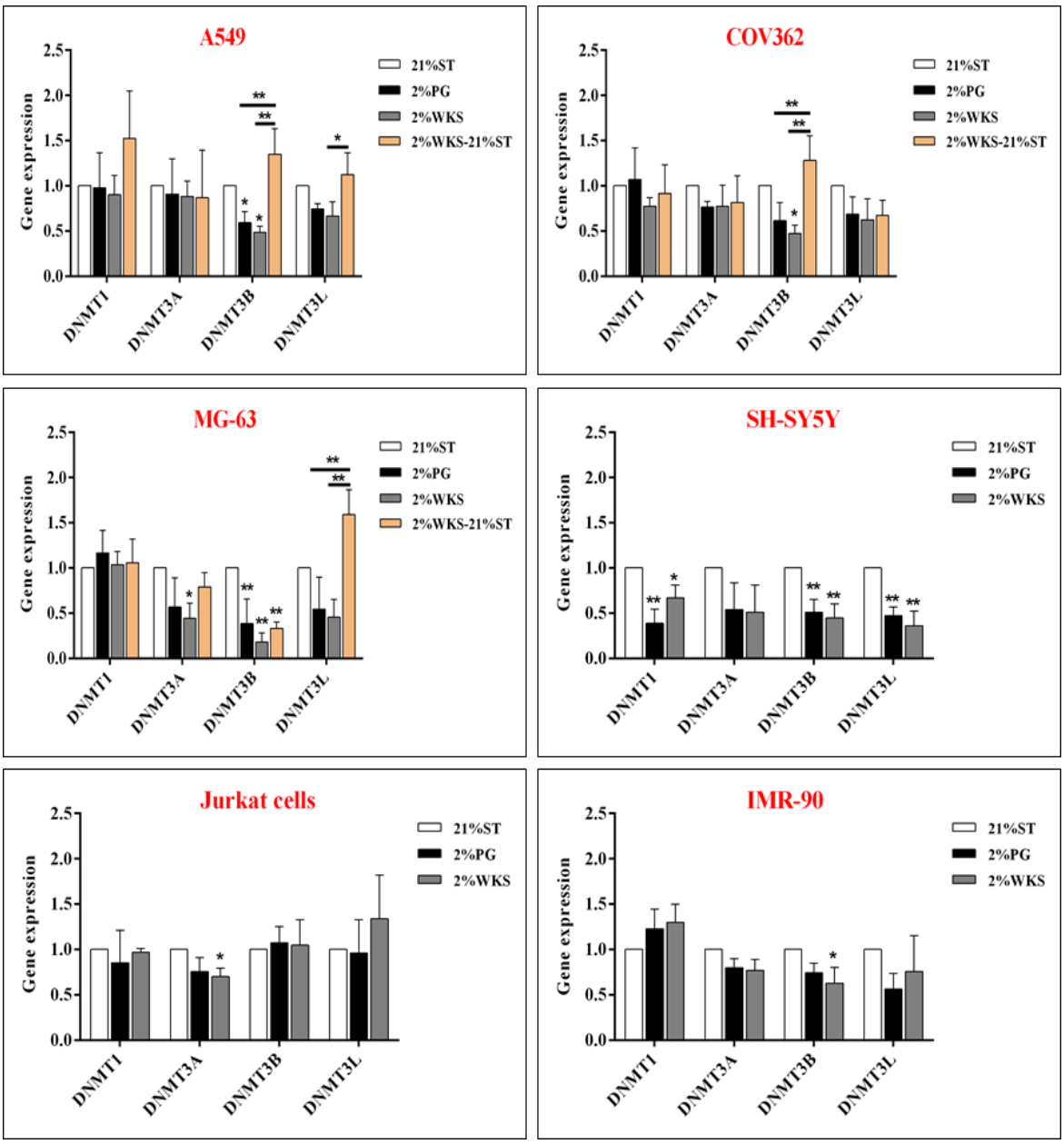


Figure 5.6 The RT-qPCR expression of the DNA methyltransferase (DNMTs) enzymes at RNA level in cancer cells. Quantification RNA showed significant decrease in DNMT3B expression in cancer cells in response to hypoxia. The expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{\wedge\Delta\Delta Ct}$ of treated cell to untreated cell. X-axis indicates DNA methyltransferase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.5.2 Tumour hypoxia increased DNA hydroxymethylation by regulated TET1 activity

We also examined TETs mRNA expression by qPCR **(Figure 5.7)**. We found that TET1 appeared to be increased in the cancer cell lines cultured in 2%PG and 2%WKS compared to the air oxygen 21%ST. The level of TET1 was increased significantly in A549, COV362 and SH-SY5Y cells following treatment with 2%WKS (2.1, 1.5 and 1.5, $P<0.05$), and in MG-63 cultured in 2%PG and 2%WKS (1.4, $P<0.05$ and 1.6, $P<0.01$) respectively, versus 21%ST **(Figure 5.7)**. In contrast, the level of TET1 was decreased significantly in non-cancer cell line (IMR-90) following treatment with 2%WKS (0.51, $P<0.05$) **(Figure 5.7)**. Similar significant reduction was observed in TET3 level in Jurkat cells grown under 2%PG and 2%WKS conditions (0.71 and 0.69, $P<0.01$), respectively versus 21%ST **(Figure 5.7)**.

In addition, a significant difference was observed in TET1 and TET3 in A549 cells cultured in 2%WKS-21%ST condition (1.1 and 0.88, $P<0.05$) respectively, in comparison to those cultured in 2%WKS conditions **(Figure 5.7)**. Similar significant differences were noted in COV362 cells cultured in 2%WKS-21%ST in comparison to those cultured in 2%PG and 2%WKS conditions. In addition, significant changes in TET2 level were observed between 2%WKS-21%ST and 21%ST (0.59, $p<0.05$) in COV362 cells **(Figure 5.7)**. No significant change was observed in TET2 and TET3 expression in cancer cells cultured in both hypoxia conditions in compared to air oxygen conditions.

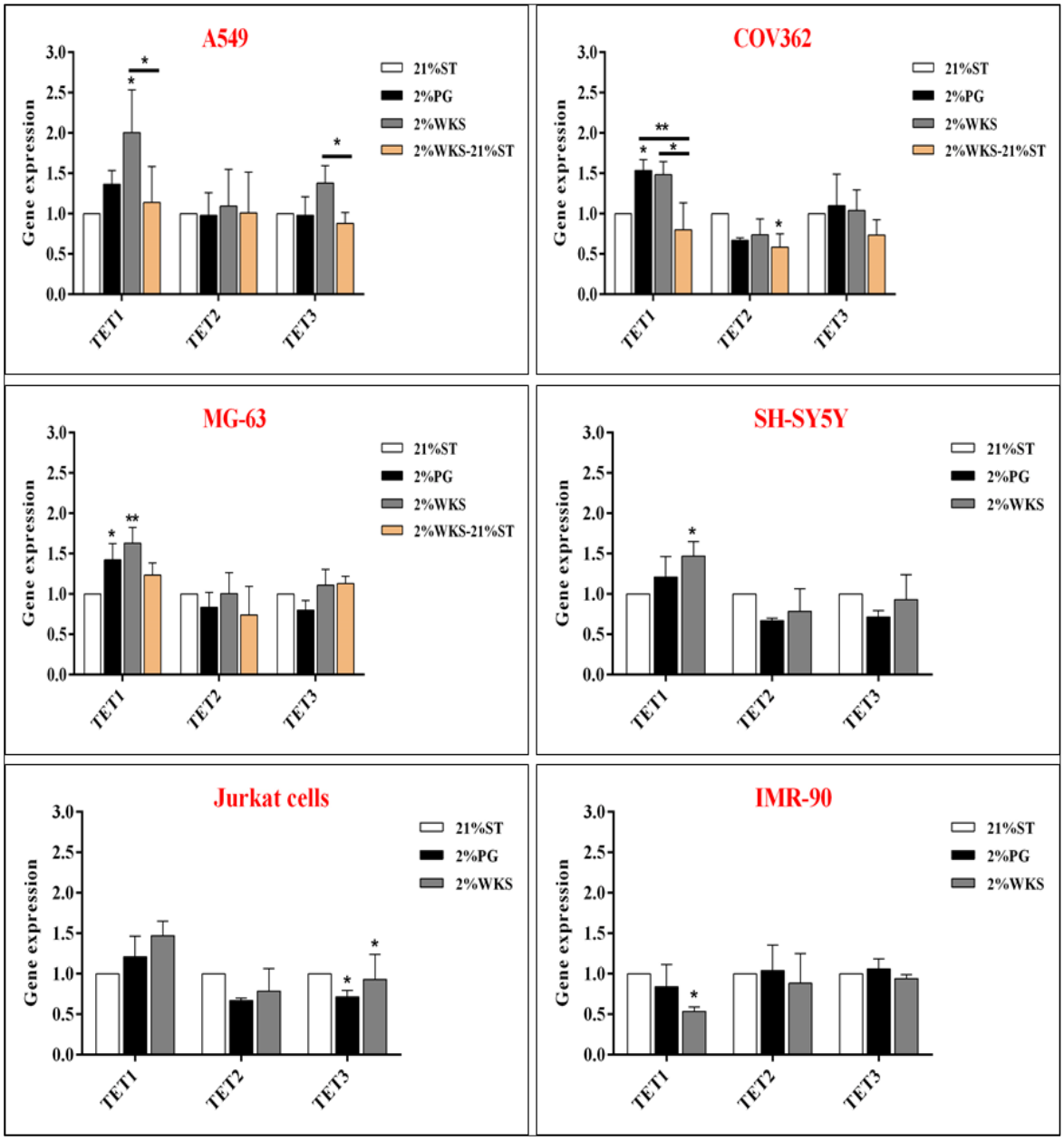


Figure 5.7 The RT-qPCR expression of the ten-eleven translocation methylcytosine dioxygenase (TETs) enzymes at RNA level in cancer cells. Quantification of mRNA showed a significant increase in TET1 expression in cancer cells in response to hypoxia. The expression was normalized to the expression of β-actin. Y-axis indicates relative changes in $2^{\wedge\Delta\Delta Ct}$ of treated cell to untreated cell. X-axis indicates ten-eleven translocation methylcytosine dioxygenase enzymes. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.4.6 Reduction in DNMT3B and increased TET1 protein expression in cancer cells subjected to hypoxia

To confirm that the decrease in DNMT3B and TET1 transcription was accompanied by down-regulation of DNMT3B and TET1 at protein level we used western blot analysis on protein isolated from cancer cell lines in hypoxia conditions compared to cells maintained in air oxygen conditions. Profiling of protein expression showed that expression of DNMT3B and TET1 protein in cancer cells cultured in varing oxygen conditions in a broadly similar pattern to that observed with mRNA expression **(Figure 5.8)**.

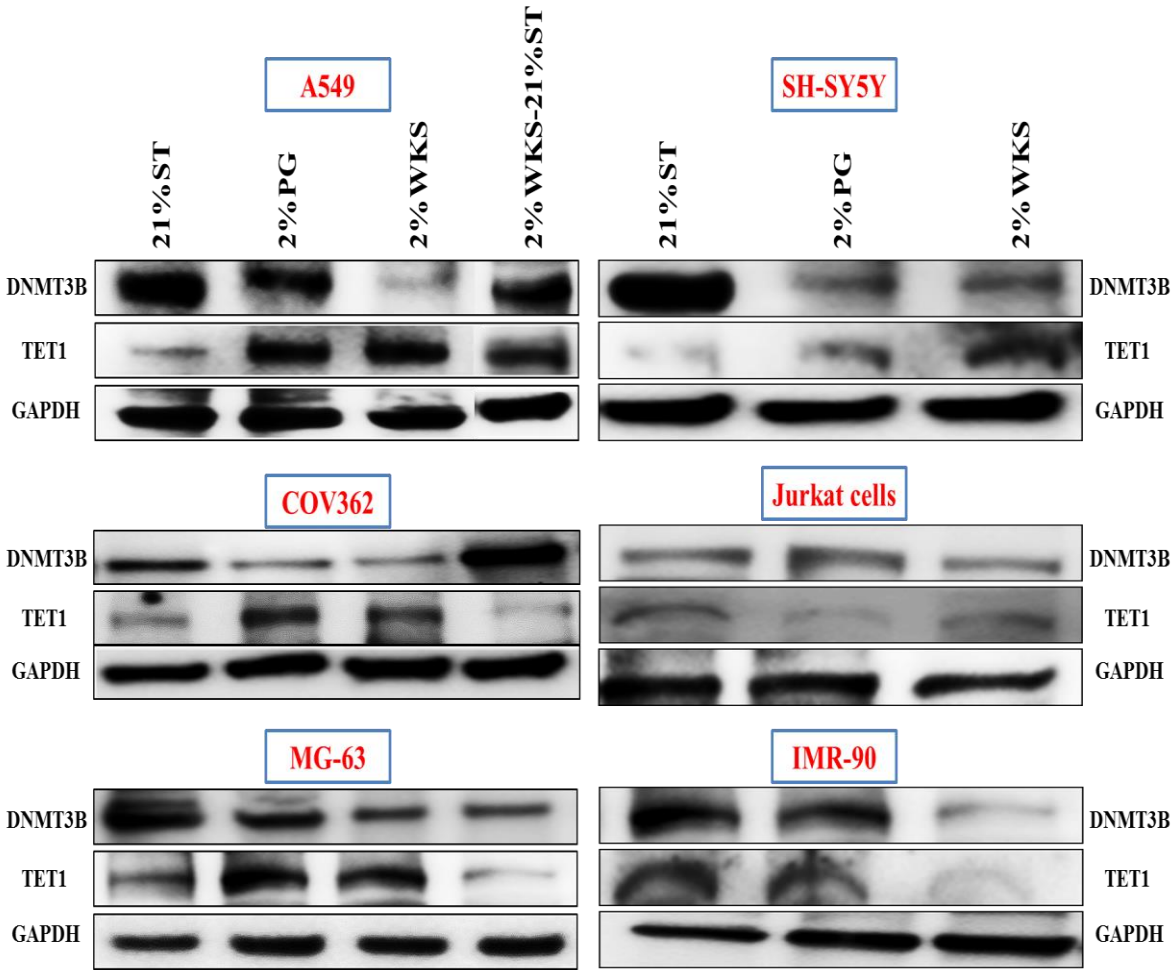


Figure 5.8 Changes in DNMT3B and TET1 expression in cancer cells, at protein levels measured by immunoblotting. Protein was isolated from cancer cells following incubation in varying oxygen tensions. GAPDH was used as a loading control.

5.4.7 Hypoxia induces DNMT3B promoter hypermethylation levels in cancer cells

To confirm that the differences in mean levels of DNA methylation between different oxygen tensions at both transcript and protein levels in cancer cells **(Figure 5.4.5)** are consistent with methylation at promoter level, the DNA methylation status of seven gene promoters at the CpGs level was determined by using pyrosequencing. The results showed significantly greater mean levels of DNMT3B promoter methylation in A549 cells following DNA isolation from 2%PG and 2%WKS (25.25% and 27%, respectively) when compared with air oxygen (13.5%) **(Figure 5.9)**. Similar results were observed in COV362 cultured in 2%PG and 2%WKS (48.5% and 56%, respectively) when compared with air oxygen (8%), and in MG-63, SH-SY5Y and IMR-90 cells following treatment with 2%WKS (41.25%, 26.75 and 23.25%) respectively, versus 21%ST (10.25%, 13% and 7%, respectively) **(Figure 5.9)**.

Interestingly, a significant difference in mean promoter methylation levels of DNMT3B in A549 and MG-63 cells cultured in 2%WKS-21%ST condition (16.75% and 15.25) respectively, was observed in comparison to those cultured in 2%WKS **(Figure 5.9)**. Significant differences were also noticed in COV362 cells cultured in 2%WKS-21%ST (24%) in comparison to those cultured in 2%PG and 2%WKS conditions. We also observed a significant reduction in mean promoter methylation levels of DNMT3L in A549, COV362 and MG-63 cells cultured in 2%WKS-21%ST (46.3%, 54% and 54.3) respectively, when compared to 21%ST (61%, 81.5% and 80.5%) (**Figure 5.9)**. Similar significant differences were observed in mean promoter methylation levels of A549, COV362 and MG-63 cells that cultured in 2%WKS-21%ST when compared to both hypoxia conditions (2%PG and 2%WKS). However, no significant change was observed in the relative promoter methylation levels DNMT1 and DNMT3L in cancer cells cultured in all oxygen conditions **(Figure 5.9)**.

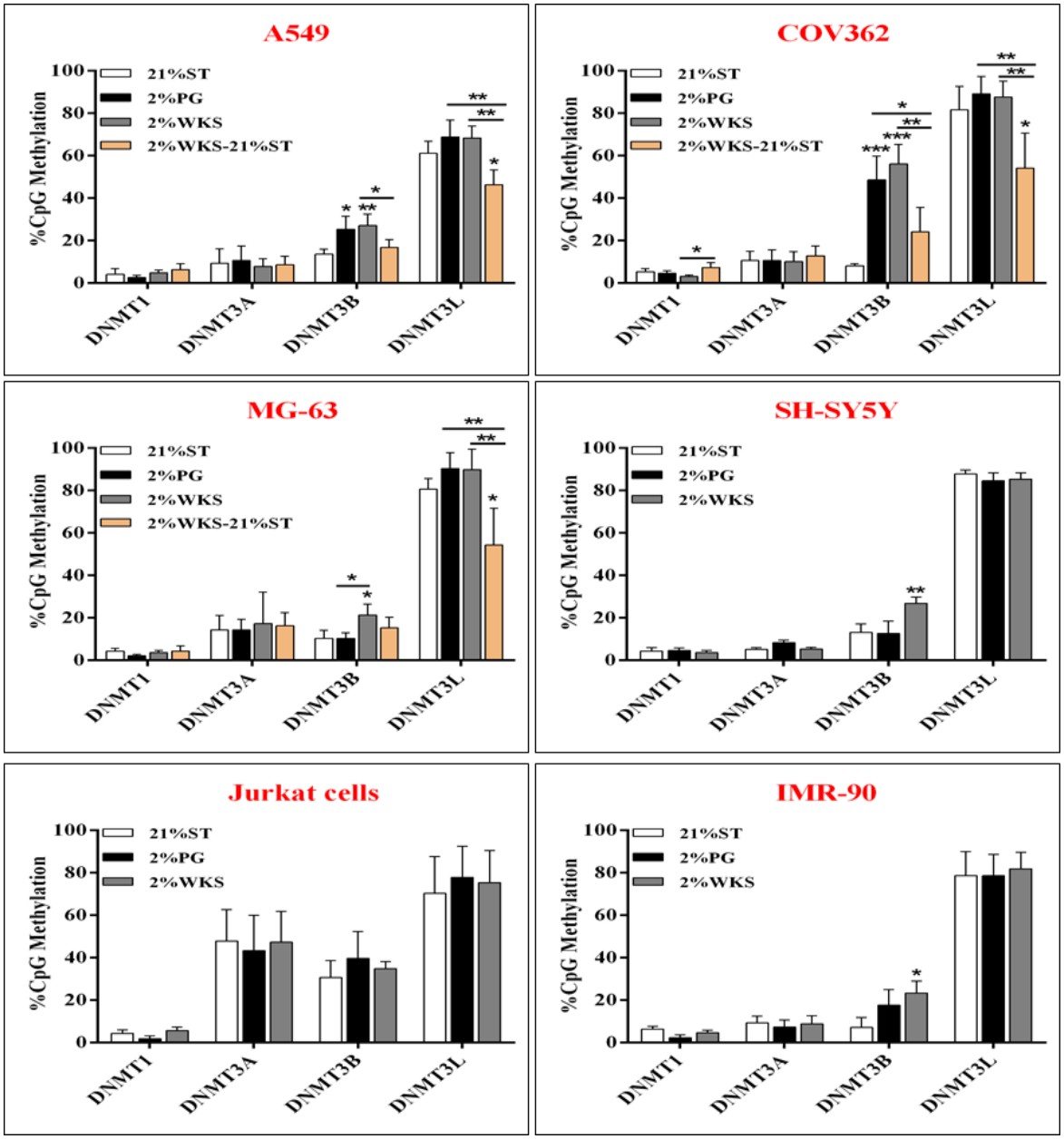


Figure 5.9 Mean levels of promoter methylation in hypoxia relative to air oxygen in human cancer cell lines. A set of DNMTs genes promoter were evaluated using pyrosequencing. Hypoxia induces DNMT3B promoter hypermethylation levels in cancer cells. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates DNA methyltransferase enzymes. Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001 vs air oxygen (21%ST).

We next investigated the effects of hypoxia at promoter methylation level of TETs. However, quantitative analysis of methylation percentage revealed no statistically

significant difference among four groups of oxygen excepted the mean promoter methylation levels of TET1 in A549 and MG-63 cells that returning to 2%WKS-21%ST condition when compared to hypoxia conditions as shown in **Figure 5.10**.

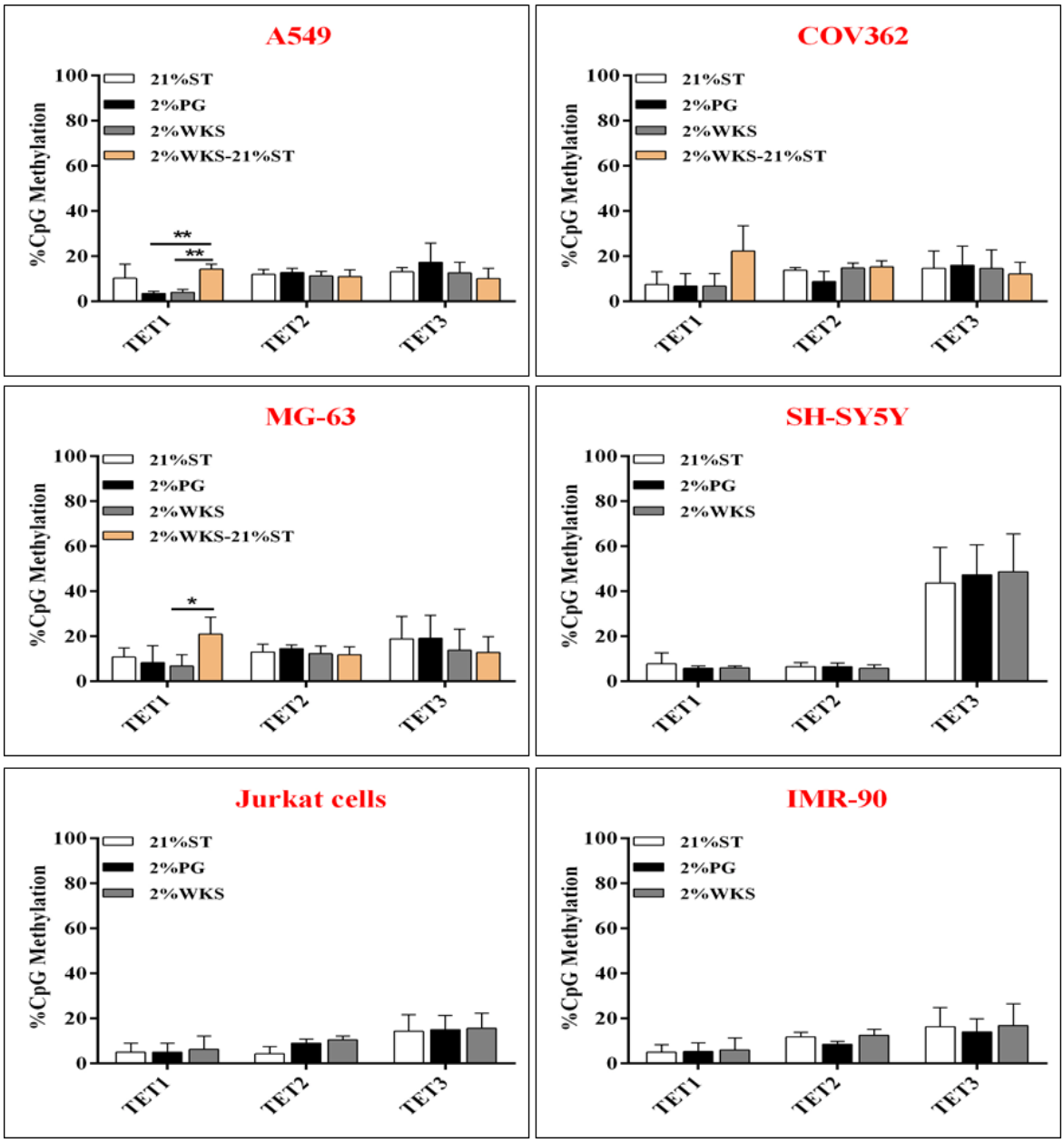


Figure 5.10 Mean levels of promoter methylation in hypoxia relative to air oxygen in human cancer cell lines. A set of TETs genes promoter were evaluated using pyrosequencing. Y-axis indicates DNA methylation levels at CpG regions. X-axis indicates ten-eleven translocation methylcytosine dioxygenase enzymes. Data are presented as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001.

5.4.8 HIF2A protein expression by Western blotting was increased in cancer cells subjected to hypoxia

To identify the effects of reduced oxygen incubation on the HIFs expression profiles, transcriptional and protein levels of HIF1A and HIF2A in human cancer cell lines cultured in different oxygen conditions were analysed with qPCR and Western blotting assays. Results showed there was significantly less relative gene expression of HIF1A (0.36 ± 0.13) in A549 cells cultured in 2%WKS-21%ST condition when compared to those cultured in air oxygen (21%ST), and when compared to 2%PG and 2%WKS (0.82 ± 0.17 and 0.71 ± 0.11, respectively). In contrast, no significant change was observed in the level of mRNA HIF2A expression in all oxygen conditions **(Figure 5.11A)**. The protein level of HIF2A (2.3 ± 0.23 and 2.4 ± 0.43, respectively) was significantly elevated in A549 cells following treatment with 2%PG and 2%WKS when compared to 21%ST **(Figure 5.11B)**. In addition, a significant difference was observed in HIF2A in A549 cells that cultured in 2%WKS-21%ST condition (1.6 ± 0.38) in comparison to those cultured in 2%WKS conditions. No significant change in HIF1A protein was observed in all oxygen conditions **(Figure 5.11B)**.

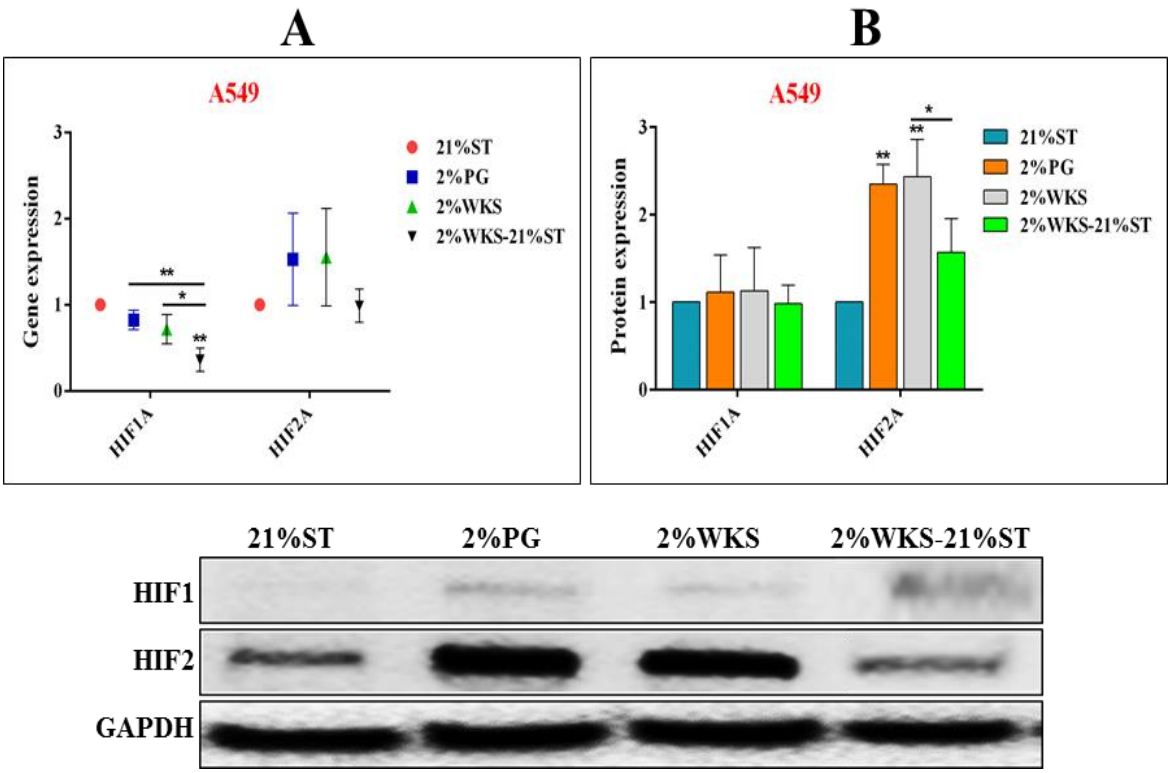


Figure 5.11 The level of HIFs expression in A549 cells cultured in different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

We noted significantly less relative gene expression of HIF1A (0.53 ± 0.19, 0.55 ± 0.06 and 0.49 ± 0.13), respectively in COV362 cells cultured in 2%PG, 2%WKS and 2%WKS-21%ST conditions in comparison to those cultured in air oxygen (21%ST). A significant increase in the gene expression levels of HIF2A was noticed in COV362 cells cultured in 2%WKS (1.5 ± 0.27) in comparison to those cultured in air oxygen (21%ST) **(Figure 5.12A)**. In addition, a significant difference was observed in HIF2A in COV362 cells that cultured in 2%WKS-21%ST condition (0.79 ± 0.19) in comparison to those cultured in 2%PG and 2%WKS conditions (1.4 ± 0.14 and 1.5 ± 0.27), respectively **(Figure 5.12A)**.

A significant increase in HIF2A protein expression was noted in 2%PG and 2%WKS conditions in COV362 cells (2.24 ± 0.63 and 2.44 ± 0.36) respectively, when compared with air oxygen **(Figure 3.12B)**. Also, a significant difference was observed in HIF2A in COV362 cells that cultured in 2%WKS-21%ST condition (1.3 ± 0.31) in comparison to those cultured in 2%WKS conditions **(Figure 3.12B)**. HIF1A protein was not detected in BMA-20 cells.

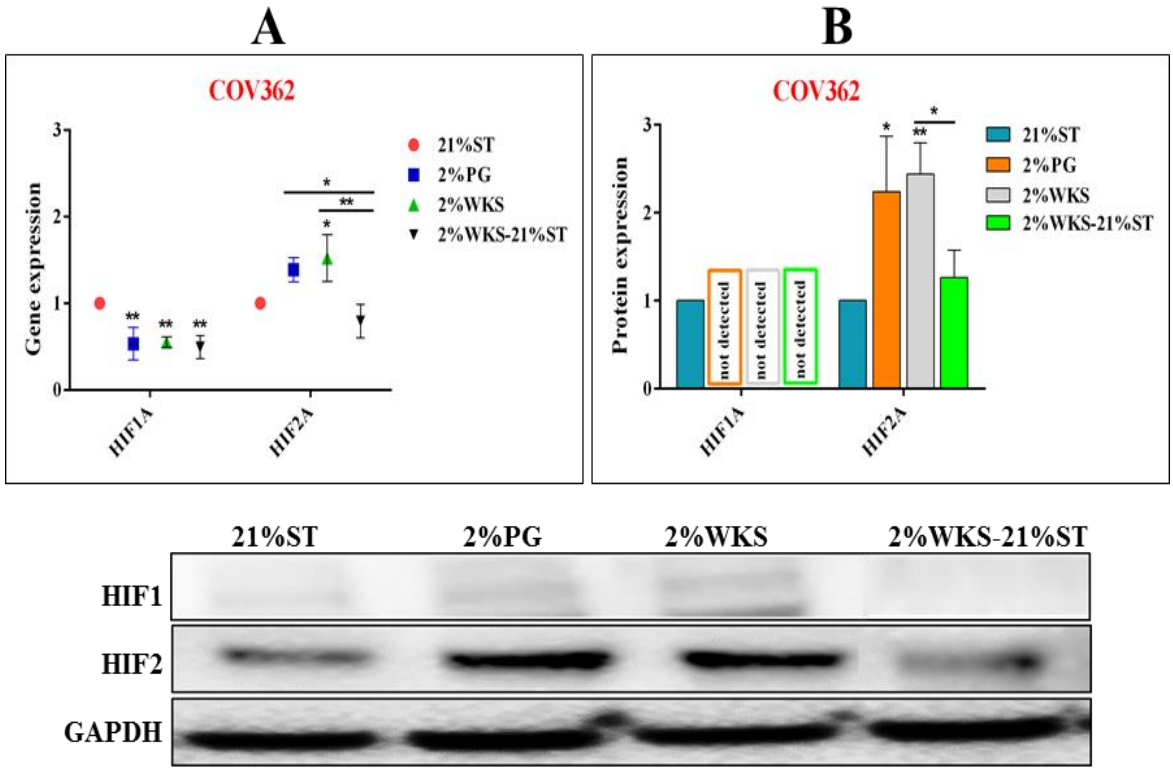


Figure 5.12 The level of HIFs expression in COV362 cells cultured in different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

The results of MG-63 showed there was significantly less relative gene expression of HIF1A (0.46 ± 0.13 and 0.45 ± 0.14) respectively, in MG-63 cells cultured in 2%PG and 2%WKS condition when compared to those cultured in air oxygen (21%ST), and when compared the

2%PG and 2%WKS with 2%WKS-21%ST (0.8 ± 0.15) **(Figure 5.13A)**. In contrast, no significant change was observed in the level of mRNA HIF2A expression in all oxygen conditions **(Figure 5.13A)**. The protein level of HIF2A (2.03 ± 0.3) was significantly elevated in MG-63 cells following treatment with 2%PG when compared to 21%ST, and when the 2%PG compared with 2%WKS and 2%WKS-21%ST (1.8 ± 0.3 and 0.8 ± 0.21) respectively **(Figure 5.13B)**. HIF1A protein was not detected in MG-63 cells.

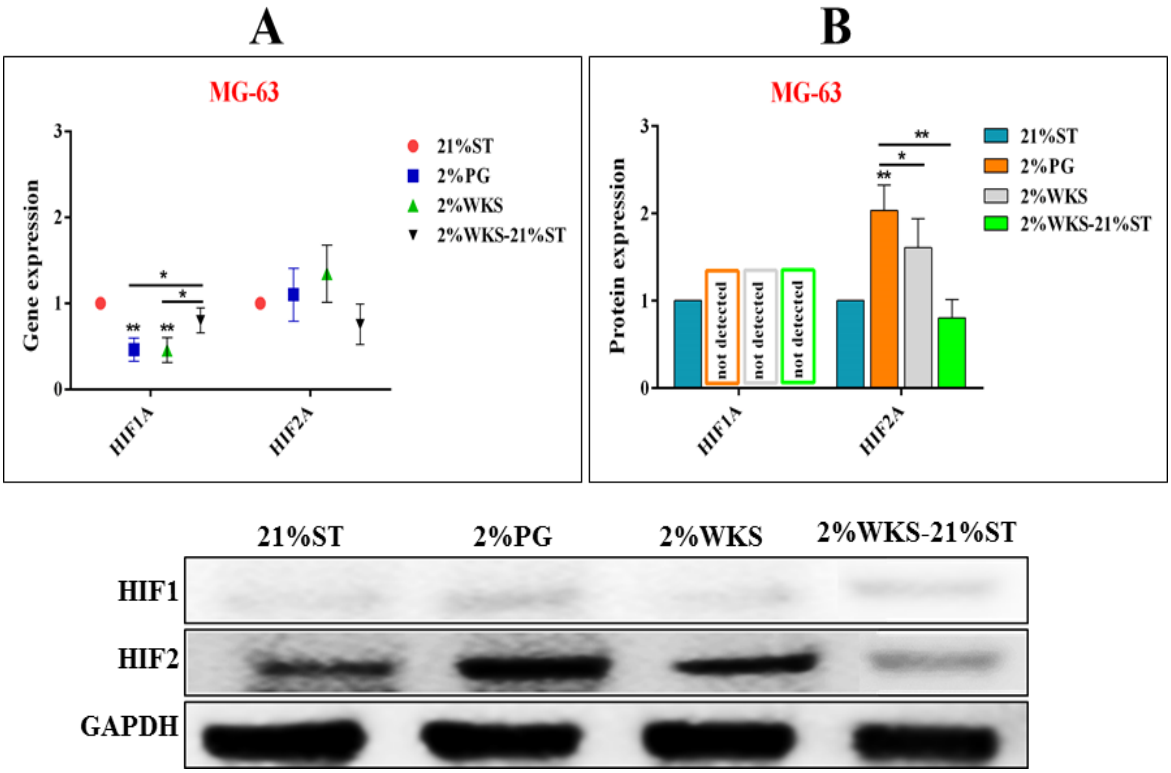


Figure 5.13 The level of HIFs expression in MG-63 cells cultured in different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

Similarly, significantly less HIF1A gene expression (0.53 ± 0.2 and 0.58 ± 0.09, respectively) was noted in SH-SY5Y cells grown under 2%PG and 2%WKS conditions when compared with the air oxygen **(Figure 3.14A)**. The level of HIF2A was increased in SH-SY5Y cells cultured under 2%PG and 2%WKS but no significant change was observed

when compared to air oxygen **(Figure 3.14A)**. HIF2A protein (2.1 ± 0.12 and 2.05 ± 0.42, respectively) was increased significantly in SH-SY5Y cells cultured under 2%PG and 2%WKS when compared with air oxygen **(Figure 3.14B)**. No significant change in HIF1A protein was observed in all oxygen conditions.

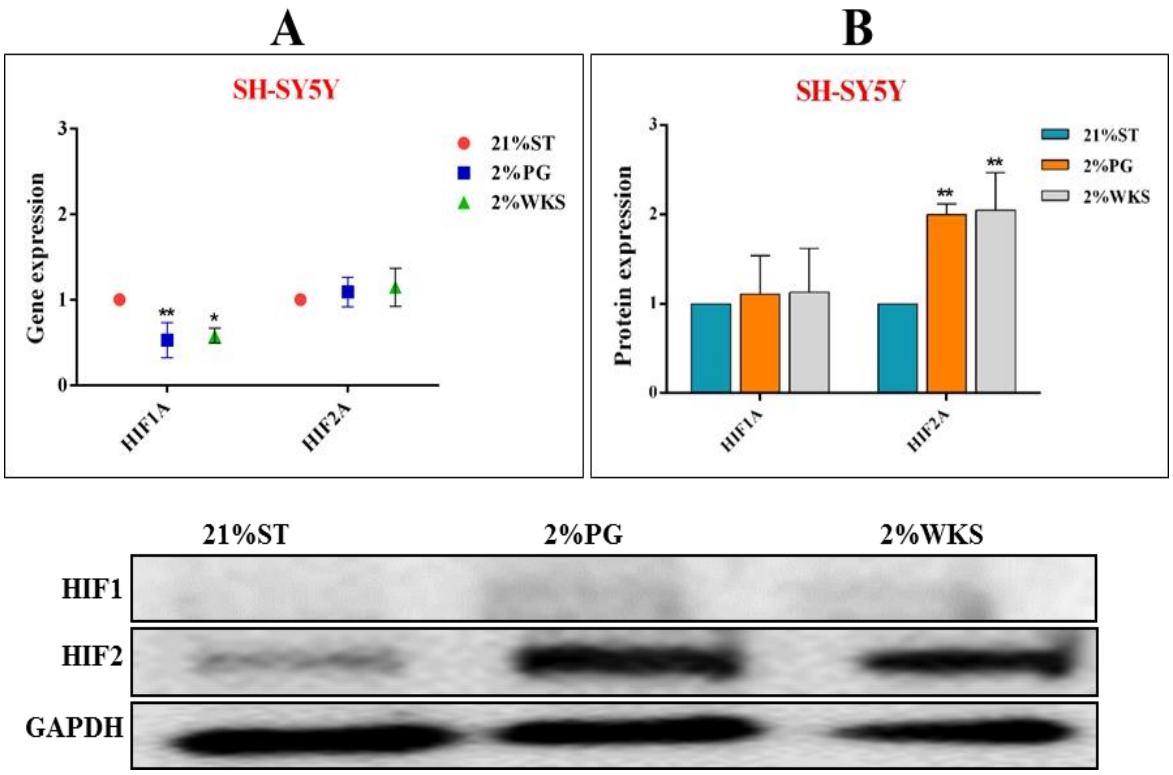


Figure 5.14 The level of HIFs expression in SH-SY5Y cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

In addition, there was no significant change in HIF1A and HIF2A at transcriptional and protein levels in Jurkat cells that cultured in different oxygen conditions **(Figure 3.15A and B)**.

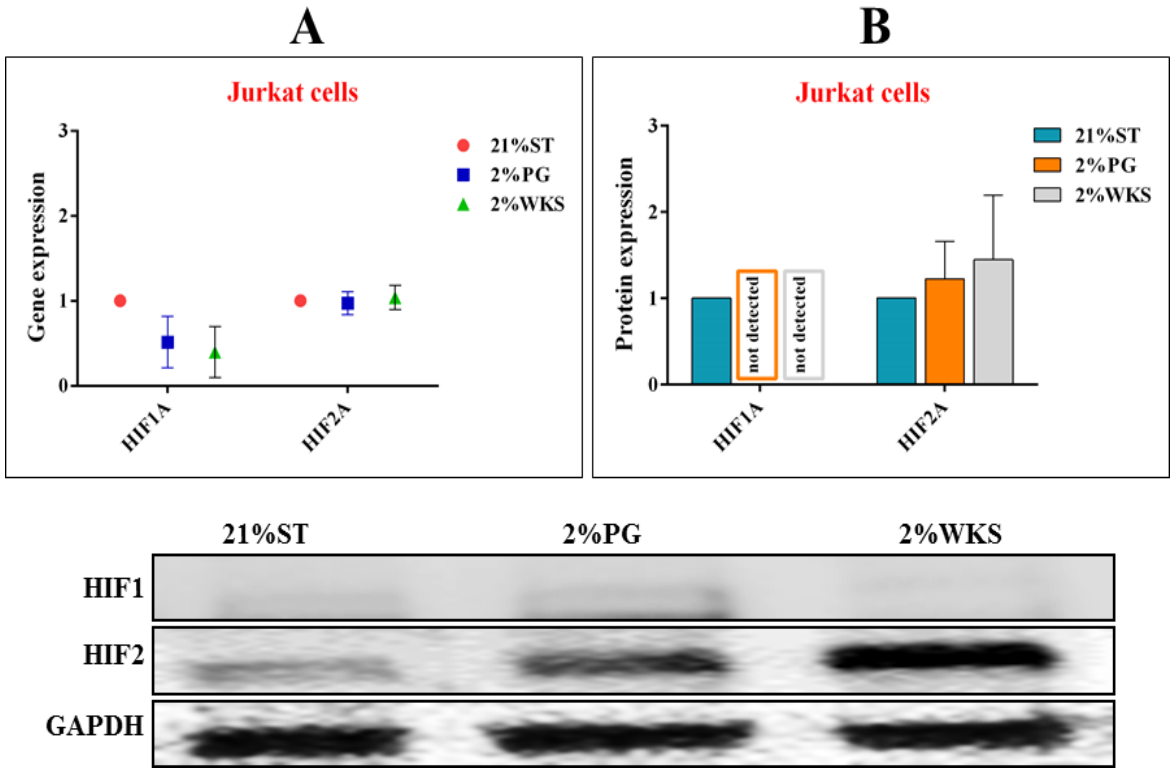


Figure 5.15 The level of HIFs expression in Jurkat cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3.

On the other hand, the results showed a significant reduction in gene expression in IMR-90 cells that cultured in 2%PG and 2%WKS (0.5 ± 0.3 and 0.4 ± 0.3, respectively) when compared to those cultured in air oxygen **(Figure 5.16A)**. Also, the level of HIF2A was increased in SH-SY5Y cells cultured under 2%PG and 2%WKS but no significant change was observed when compared to air oxygen **(Figure 3.16A)**. The protein level of HIF1A and HIF2A (1.8 ± 0.38 and 1.3 ± 0.15, respectively) was significantly elevated in MG-63 cells following treatment with 2%PG when compared to 21%ST **(Figure 3.16B)**.

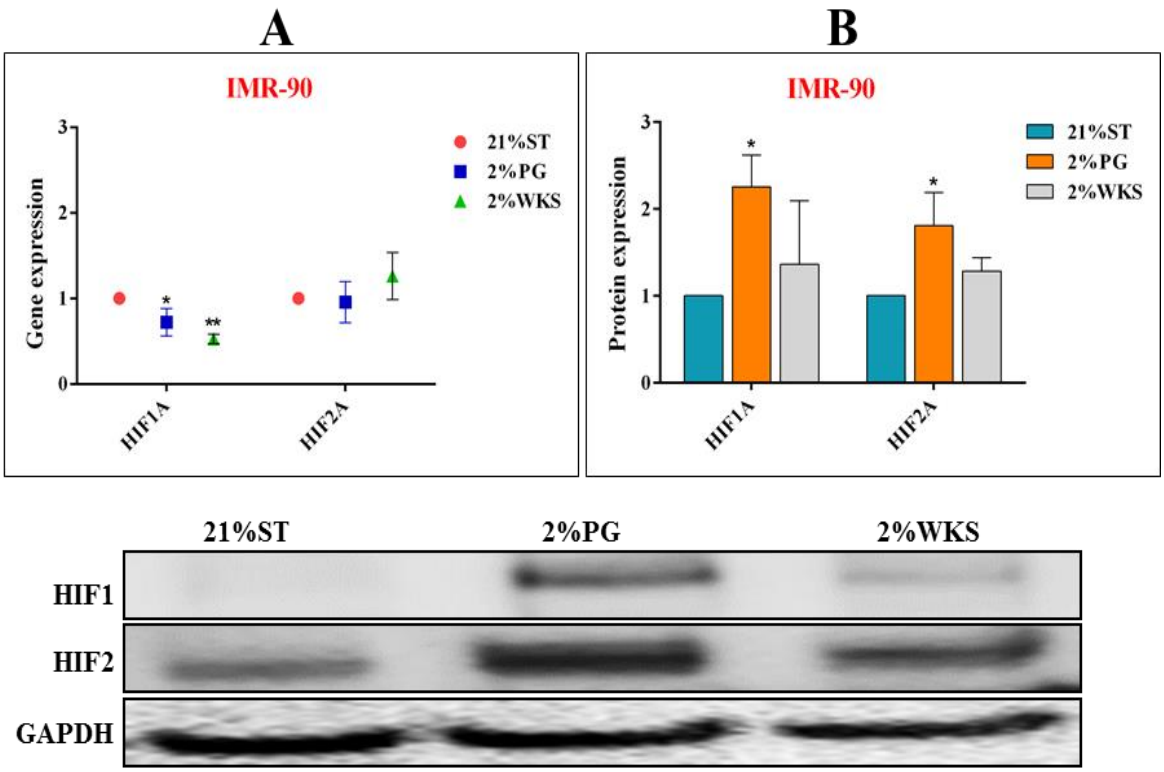


Figure 5.16 The level of HIFs expression in IMR-90 cells cultured in three different oxygen conditions. A) The RT-qPCR expression of the HIFs normalized to the expression of β-actin. B) Western blot analysis of HIFs normalized to the expression of GAPDH and to 1 for 2% oxygen. Data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01 vs air oxygen (21%ST).

5.5 Discussion

Over the last two decades, extensive research into cancer epigenetics has come to challenge the long-standing view of genetic mutations as the key determinant in tumourigenesis (Sharma et al., 2010, Baylin and Jones, 2011). Discoveries of abnormalities in global methylation patterns within cancer cells and its effects on gene expression independent of genetic mutations has highlighted a role of epigenetics in cancer development (Sharma et al., 2010). In contrast, little is known about the impact of changes within the tumour microenvironment on the global DNA methylation of cancer cells, particularly hypoxic stress. Therefore, in this chapter we aimed to understand how local hypoxic

microenvironments can alter cancer cells, triggering abnormal DNA methylation profiles *in vitro*.

The proliferation of the human cancer cell lines was explored following exposure to different oxygen conditions. We observed a significant decrease in the rate of proliferation of cancer cells cultured in both hypoxia conditions **(Figure 5.1)**. In addition, our MTT results displayed similar effect to growth rate **(Figure 5.2)**. This data is consistent with previous studies which have found that cancers cells lost their proliferation ability within hypoxic conditions as a result of the modulation of a number of metabolic mechanisms, cell cycle regulation as well as development of epigenetic modifications (Gardner and Corn, 2008; Pal et al., 2010; Xu et al., 2016). The correlation between proliferation of cancer cells and abnormal DNA methylation profiles have been examined in several cancer models (Cui et al., 2002; Shahrzad et al., 2007; Gossage and Eisen, 2010). It has been reported that disruptions of VHL (von Hippel–Lindau) through hypermethylation of its corresponding promotor region in cancer cells results in the up-regulation of hypoxia-inducible factors (HIFs) which in turn affect the cellular proliferation and survival. (Gossage and Eisen, 2010).

We observed that reduced oxygen did not lead to changes in the mRNA levels of NANOG and SOX-2, significantly. In contrast, the expression of OCT-4 was significantly increased in 2% O_2 hypoxia compared to air oxygen **(Figure 5. 3).** These results are in line with previous studies that showed that HIFs induce human pluripotency markers in cancer cells (Covello et al., 2006; Mathieu et al., 2011). Covello *et al* revealed that HIF2A, but not HIF1A, binds the OCT-4 promoter directly and activates the expression of OCT-4 which correlated to enhanced levels OCT-4 protein (Covello et al., 2006). In addition, it has been suggested that increased of OCT-4 expression in cancer cells under hypoxia conditions were associated with partial unmethylation of the promoter of OCT4 (Mathieu et al., 2011).

Next, we went onto further explore the effects a hypoxic microenvironment on global 5mC and 5hmC levels in cancer cells. We did observe an overall decrease in the global 5mC levels when cultured in hypoxic conditions (**Figure 5.4)**. Consistent with our findings, genomic hypomethylation of 5mC has been observed in both solid tumours such as hepatocellular and cervical cancer, in addition to haematological cancers such as chronic B-cell lymphocytic leukaemia (Wahlfors et al., 1992; Kim et al., 1994; Sadikovic et al., 2008). In addition, hypoxia induces genomic DNA hypomethylation through the activation of HIF1A in *vivo* (Liu et al., 2011). Furthermore, the recent discovery showed the that methylation level was decreased statistically in systemic sclerosis (SSc) fibroblasts cells (Hattori et al., 2015).

We also observed an increase in the global 5hmC levels when incubated at 2% hypoxia conditions **(Figure 5.5)**. Our results agree with previous reports that demonstrated that the level of 5hmC increased in tumorigenic N-type neuroblastoma cells exposed to 1% O_2 hypoxia (Mariani et al., 2014). In addition, Wu *et al* found that reduced oxygen led to an increased level of global 5hmC in breast cancer MCF7, MDA‑MB‑231 cell lines, as well as in primary breast cancer cells (Wu et al., 2015). Hepatoblastoma HepG2 cells cultured in 1% oxygen for 24 h, had greater levels of 5hmC than those in cells cultured in 21% oxygen (Lin et al., 2017).

To examine the relationship between the global 5mC and 5hmC patterns and the level of expression of DNMTs and TETs, we used RT-PCR and Western blot to explore the expression of these genes within cancer cells in response to hypoxia. Our results showed a decrease in the relative expression of de novo methylation DNA methyltransferase DNMT3B and DNMT3L in response to hypoxia. Notably, this may correlate to the decrease in global 5mC levels observed in cancer cells. Interestingly, this decrease was associated with significant increases in the methylation levels of these genes in cancer cells cultured

under hypoxia conditions. However, it has been reported that increased mRNA expression of DNMT3B in glioma tumours may have resulted from hypomethylation of its promoter regions (Rajendran et al., 2011). In addition, overexpression of the all three DNMTs genes resulted in DNA hypermethylation in different cancer cell lines such as stomach, colorectal carcinoma, hepatocellular and breast (Sun et al., 1997; Kanai et al., 2001; Girault et al., 2003). Also, a significant decrease in mRNA levels for DNMT1, DNMT3a and DNMT3b has been shown in human colorectal cancer cells cultured under hypoxia conditions (Skowronski et al., 2010). Similarly, Waston *et al* found that DNA methylation levels altered in cardiac tissue fibrosis with HIF1A regulating the expression of DNMT1 and DNMT3B (Waston et al., 2014). In addition, exposure to severe hypoxic conditions in *in vivo* xenograft cancer, resulted decrease in 5mC levels (Shahrzad et al., 2007). Study of all three DNMTs in Hepatoma cells has shown that low oxygen upregulated the expression of DNMT1 and DNMT3A but not DNMT3B (Liu et al., 2011). This changes in DNMTs expression could be explain via hypoxia inducible factor (HIFs) pathway, which acts through the binding of HIFs with specific hypoxia response elements (HRE) present in promoter and enhancer regions of hypoxia responsive genes. This is consistent with the fact that the flanking regions of HRE are highly variable and critical for HRE function (Kaluz et al., 2008; Liu et al., 2011; Geo et al., 2017).

We found a significant increase in the expression of the ten-eleven translocation methylcytosine dioxygenase TET1. As this is one of the enzymes responsible for the conversion of 5mC to 5hmC, the increase of its expression by cancer cells in response to hypoxia may correlate to the increase in the global 5hmC levels observed in this experiment. However, this increase in TET1 levels in hypoxic tumour cells has not revealed any association with its promoter methylation status. Low oxygen has been linked to increase the TET1 expression in cancer cells, which catalyses the conversion the 5mC to 5hmC

(Mariani et al., 2014). Similarly, two recent studies showed that the increased of 5hmC levels in cancer cells following exposure to severe hypoxic conditions was associated with up-regulation of TET1 and TET3 (Wu et al., 2015; Hattori et al., 2015). In addition, other studies have found a decrease in the expression of all three TETs in association to a decreased level of 5-hmC in human breast, liver, pancreatic and prostate cancer when compared to surrounding tissues (Yang et al., 2013). This highlights the importance of further research into the expression of TETs and its correlation to DNA methylation in cancer pathogenesis.

On the other hand, the present study demonstrates that HIF2A protein expression by Western blotting was increased in cancer cells subjected to hypoxia **(Section 5.4.8)**. This agrees with results that have shown the expression of HIF2A at protein level increases significantly when cells are cultured for long term under hypoxic condition (Gordan et al., 2007; Forristal et al., 2010). However, Cameron et al suggested that HIF1A protein is only transiently expressed for two days after exposure to hypoxia (Cameron et al. 2008). Our results also in agreement with Forsyth et al and Westfall et al results that showed there was no significant increase in the mRNA expression of the HIFs subunits under low oxygen tensions (Forsyth et al., 2008; Westfall et al., 2008). This finding suggesting that changes at the protein levels could be due to posttranscriptional regulation (Huang et al. 1998; Lang et al. 2002).

Finally, we explored the effect of modulating hypoxia on levels of DNA methylation (5mC and 5hmC) and the expression of DNMTs/TETs. We found that removal hypoxia caused significant changes in the level of 5mC and 5hmC and their DNMTs/TETs when compared to 2% hypoxia conditions in HIF2A-dependent regulation. However, RNA-Seq showed that about 369 genes were changes significantly in the hypoxic cells when the effect of hypoxia stress was removed in compared to control cells (Hartley et al., 2013). Interestingly, we found that the transition between hypoxia and normoxia induced changes in the level of DNMT3L at mRNA and methylation levels. This recent result strongly supports the

hypothesis that DNMT3L plays a role in alter DNA methylation and potential role of reduced oxygen in regulation the DNMT3L expression (Heo et al., 2017).

In conclusion, this study has highlighted the significant effect a hypoxic microenvironment can have on epigenetic changes within cancer cells as well as the importance of further research into the expression of DNMTs/TETs and its correlation to DNA methylation in cancer pathogenesis.

Keele University

Chapter 6: Summative discussion, conclusions, and further work

6.1 Summative discussion

Low oxygen induces the activation of several pathways within the cell, some being HIF-mediated and some HIF-independent. Many studies have reported that reduced oxygen is an essential factor in the maintenance, proliferation, differentiation and functions of stem cells (Yin and Li, 2006; Abdollahi et al., 2011; Zhi et al., 2018). At the same time, a significant progression has been considerable in understanding the molecular mechanisms responsible for the hypoxic tumour microenvironment that has opened a new area in the discovery of potential targets for cancer treatment (Adamaki et al., 2012). Also, many studies have linked reduced oxygen to increased epigenetic instability, which may also contribute to cancer development (Perez-Perri et al., 2011; Chen and Sang, 2012). However, whereas many investigators agree that the low oxygen tension promotes proliferation, inhibits senescence, and maintains properties of several cells including embryonic stem cells and mesenchymal stem cell but data on the effect of low oxygen culture conditions on genomic stability are rare and conflicting (Yin and Li, 2006; Bentivegna et al., 2013). However, enhanced knowledge of enzymatic function and the role of hypoxia in control the DNA methylation present new approaches for arising therapeutic strategies designed to reverse transcriptional aberrations that are inherent to the cancer epigenome (Baylin and Jones, 2012).

The principal aim of this thesis was to identify epigenetic changes potentially associated with the changes in oxygen level in different cell lines including stem cells and cancer. It was, however, essential to first study the effect of low oxygen in various functions of cells such as proliferation, metabolic activity and differentiation. In addition, the study of functional characterisation of cells was vital to ensure the quality and status of the cells before the survey these investigations. In this study, two different oxygen conditions (2%PG and 2%WKS) were used to describe the 2% oxygen culture where mimics the native oxygen level *in vivo* environment. The 21%ST was used to describe the 21% air oxygen condition

that is used in many standard laboratories culture as described in **chapter 2**. Flow cytometry technique, fluorescence image-based analysis and the tri-lineage differentiation potential showed that both stem cells (hPSCs and BM-hMSCs) have typical characteristics of the surface antigen of stem cells along with typical results of positive and negative of tri-lineage differentiation of BM-hMSCs. Also, there was no significant difference between low oxygen and air oxygen condition. These results agree with many studies that showed the low oxygen tension did not affect the typical characterisation of stem cells during normal passaging (Forsyth et al, 2006; Forristal et al., 2010; Basciano et al., 2011; Narva et al., 2013). *In vitro* studies, it is important to confirm the pluripotent nature of our cells before the continue to further investigations. Furthermore, the expression of pluripotent markers OCT-4, NANOG, SSEA-4 and ALP gradually reduced over the time course of hPSCs differentiation which indicates that cells gradually lost their pluripotency across all time points.

As early as 1958, Cooper *et al* found that animal cells grow more rapidly in low oxygen tension than air oxygen (Cooper et al., 1958). Since that time, many researchers have studied the effect of low oxygen in proliferation and metabolic activity of several cells including stem cells and cancer (Forristal et al., 2010; Rajala et al., 2011; Xu et al., 2016). However, A variety of studies showed that cells grow more efficiently under reduced oxygen conditions when compared to air oxygen (Ivanovic et al., 2000; Studer et al., 2000). However, our results showed that physiological normoxia conditions (2%PG and 2%WKS) increased the proliferation and metabolic activity of hPS cells as compared with air oxygen. On the other hand, hypoxia treated cancer cells showed distinct changes in proliferation and metabolic activity. The variability of these differing responses of cells to reduced oxygen may be due to the different oxygen concentrations that used during experiments as well as the cell type (Abdollahi et al., 2011). Moreover, Heyman *et al* reported that different cells types shown the distinct ability to cope with reduced oxygen during a long term of culture.

In addition, the degree of their adaptive capacities may reflect their native oxygen microenvironments. (Heyman et al., 2012). However, it has reported that changes of HIFs stabilisation under different oxygen levels may participate in control the activity of some genes that involved in regulate of mitochondrial oxidative metabolism such as pyruvate dehydrogenase complex (PDC) (Semenza, 2007; Natarajan and Becker, 2012). Interestingly, the changes in proliferation and metabolic activity that we found was associated with changes in 5hmC but not 5mC. However, Bachman *et al* reported that increases in cell proliferation might be due to reducing of 5hmC level (Bachman et al., 2014). Recently, Zhu *et al* found that knockdown of TET1 led to decrease the migration, invasion and proliferation of the trophoblast-like cell line JEG3 exposed to low oxygen concentration (Zhu et al., 2017). On the other hand, hypermethylation of TET1 did not affect the proliferation rate of cells that culture under hypoxia conditions (Thienpont et al., 2016). However, further study is needed to delineate the effects of DNA methylation on proliferation and metabolic activity of cells under low oxygen conditions.

Next, because changes in pluripotent markers such as OCT-4, NANOG and SOX-2 were relevant with the changes in oxygen concentrations (Forristal et al., 2010), we investigated whether this was competing with DNA methylation changes. However, the study of pluripotent markers can provide central insights into the understanding of transcriptional circuitry responsible for supporting the self-renewal and stemness of stem cells (Han et al., 2014). We found that exposed our cells to low oxygen conditions (2% O_2) led to increasing the expression of OCT-4 and SOX-2 but not NANOG in most cell types investigated. These results are in line with previous studied that showed that HIFs induce human pluripotency markers in both stem cells (hPSC and hMSC) and cancer cells (Covello et al., 2006; Mathieu et al., 2011; Barbosa et al., 2012; Han et al., 2014). Covello *et al* revealed that HIF2A, but not HIF1A, binds the OCT-4 promoter directly and activates the expression of OCT-4 which

correlated to enhance the levels of OCT-4 protein (Covello et al., 2006). Importantly, we observed that the increases of pluripotent markers in our cells were associated with DNA hypomethylation, indicating that increases pluripotent markers underlie 5mC reduction. Furthermore, these results suggested that the catalytic activity of TET1 does not affect the gene activity of pluripotent markers in cancer cells. However, Tsai *et al* found that DNMT1 regulate OCT-4 and NANOG in MSC through binding to their promoter leading to the repressed the expression of genes associated with the development and lineage differentiation and maintain self-renewal and undifferentiated State (Tsai et al., 2014). In addition, it has been suggesting that increases of OCT-4 expression in cancer cells under hypoxia conditions were associated with partial unmethylation of the promoter of OCT-4 (Mathieu et al., 2011). Moreover, our re-oxygenation results suggest that oxygen can regulate the activity of cancer cells through HIF2A/ DNMT3B mediated control of OCT-4 expression. However, it has reported that inhibiting HIF2A activity can regulate stem cell function through reducing the expression of genes that involved in regulating stem cells including OCT-4 (Covello et al., 2006).

Additionally, Oxygen is an essential factor for several cellular processes in various organisms and, any alternative in its availability can produce significant changes in cellular quantities (Melvin and Rocha, 2012). Hypoxia-inducible factors (HIFs) is a crucial mediator of the adaptive cellular response to hypoxia and is involved in regulating the expression of many genes that affect cell survival and metabolism (Eliasson and Jonsson, 2010). However, HIFA consists of three isoforms: HIF1A, HIF2A and HIF3A, whereas HIF-β, also known as ARNT (aryl hydrocarbon receptor nuclear translocator), has only one isoform, HIF1β. Three alpha subunits (HIF1A, HIF2A, and HIF3A) all bind to subunit HIF1β (Gordan and Simon, 2007). Our results showed that that HIF2A protein expression by Western blotting was increasing in most our cells subjected to reduced oxygen. These results agree with previous

reports that have demonstrated the expression of HIF2A at protein level increases significantly when cells are cultured for a long term under low oxygen conditions (Gordan and Simon., 2007; Forristal et al., 2010). Recently, two studies revealed that the expression of HIF1A was significantly increased under low oxygen condition within 24 h and before they dropped down. On the other hand, the expression of HIF2A consistently remained at a high level (Cameron et al. 2008; Zhi et al., 2018). Also, different Western blotting studies indicated that HIF2A was upregulated under hypoxic conditions compared with 21% oxygen (Grayson et al., 2007; Forristal et al., 2010; Lin et al., 2011; Narva et al.,2013). This finding suggests that changes of HIFs at the protein levels could be due to posttranscriptional regulation (Huang et al. 1998; Lang et al. 2002).

However, routine culture of cells in low oxygen conditions resulted in a significant decrease in levels of 5mC and 5hmC, accompanied by transcriptional-translational down-regulation of DNMT3B and TET1 in most cell types investigated except the levels of TET1 and 5hmC in cancer cells. Interestingly, analysis of methylation status of specific gene promoters by measuring CpG promoter methylation levels indicated that low oxygen alters the DNMT3B promoter methylation levels, but not TET1. These results suggest that modest hypoxia (2% O_2) did not affect TET activity at promoter regions, indicating that TET enzymes are HIF-independent in line with previous reports (Laukka et al., 2016; Thienpont et al., 2016). In addition, these results showed that increases in 5hmC did not translate into global 5mC increases, probably because TETs do not target 5mC at many sites (Williams et al., 2011; Thienpont et al., 2016). Hoffner *et al* found that no association between 5mC and 5hmC and the reduction in 5hmC can occur independently from 5mC decrease (Hoffner et al., 2011). Many studies indicate that 5hmC is functionally distinct from 5mC and there is evidence for 5hmC being a stable derivate of 5mC, rather than just an intermediate of demethylation (Ecsedl et al., 2018).

Furthermore, our results showed there is no linking between increases the activity of TET1 at mRNA level and its promoters CpG content. These results support strongly the hypothesis that the expression of the gene depends on promoters CpG level, with methylated high CpG level promoters being usually inactive, whereas methylated low CpG level promoters can be active or inactive (Berdasco and Esteller, 2011; Bentivegna et al., 2013). Also, low oxygen reduces DNMT3B activity with accompanied 5mC in most cells' types investigated. Interestingly, decreases in the expression of DNMT3B was associated with a high methylated pattern in the CpG regions of its promoters. Consistent with our findings, genomic hypomethylation of 5mC has been observed in many cells that culture under reduced oxygen levels (Liu et al., 2011). Most importantly, cancer cells that returned from hypoxic condition to air oxygen showed that levels of HIF2A were decreased significantly compared to hypoxic conditions. Furthermore, reduces the level of HIF2A under (2%WKS-21%ST) condition was associated with the changes in DNMT3B levels at mRNA gene and their methylation CpG promoter levels. These results indicate that HIF2A is driving the changes in 5mC level through DNMT3B. However, cells maintained at reduced oxygen revealed decreases in the global levels of 5mC, whereas in the absence of HIF1A, the global level of DNA methylation increased accompanied by increases in DNMT3B expression (Waston et al., 2009; Waston et al., 2010). Skowronski *et al* reported that a possible mechanism for the observed changes in DNMTs expression might be due to the hypoxia inducible factor (HIFs) pathway, which acts through the binding of HIFs to the hypoxia response element (HRE) DNA recognition sites (Skowronski et al., 2010). In addition, changes in DNA methylation can impact the control of HIFs binding to target genes directly by methylation of a specific CpG dinucleotides within the hypoxia response elements (HRE) which are present in promoters, introns, and 3´ enhancers (Rossler et al., 2004; Waston et al., 2010).

Interestingly, we found that the transition between hypoxia and air oxygen-induced changes in the levels of DNMT3L at mRNA and its promoters methylation levels. These recent results strongly support the hypothesis that DNMT3L plays a role in altering DNA methylation and the potential role of reduced oxygen in regulation the DNMT3L expression (Heo et al., 2017). In addition, recent studies have reported that DNMT3L (DNA methyltransferase 3-like) does not possess methyltransferase activity itself but interacting with DNMT3A and DNMT3B to stimulate their catalytic activity. However, the precise function of DNMT3L in these cells is not known (Bourc'his, 2001; Margot et al., 2003).

In addition, our results showed that data of continuous hypoxic culture (2%WKS) are more consistent in compared to semi-continuous hypoxic culture (2%PG). This variation between two hypoxic conditions might due to fluctuation of oxygen at cellular level during seeding, changing media or passaging or opening and closing incubator doors that associated with 2% O2 model (intermittent hypoxia).These results are in line with previous studied that showed the fluctuations in oxygen concentration can affect cell growth, differentiation, signalling, and free radical production (Place et al., 2017). In addition, the adapt of cells to reduced oxygen over extended periods of incubation and culture is very important factors to the reduce of variations (Ahmed et al., 2016). For example, there is evidence that HIF-PHD enzymes can adapt to chronic oxygen depletion by changing their “set point” or sensitivity with regard to their response to local oxygen concentrations. However, a state of constant change (i.e. in cell culture) would preclude a new set-point from being reached and signalling through the oxygen-sensing pathways is inconstant flux (Hirsila, 2004; Koivunen et al., 2005; Abaci et al., 2010).

6.2 Conclusions

The main aim of this study was to identify the DNA methylation changes that potentially associated with the changes in oxygen level in different cell lines including stem cells and cancer. Also, to study of the effect of reduced oxygen in many functions of cells such as proliferation, metabolic activity, stemness and differentiation. The results presented in this work show that low oxygen enhances the function profiles of stem cells including proliferation, metabolic activity and stemness. In contrast, the culture of cancer cells with 2% oxygen causes the significant decrease in growth profiles in comparison to air oxygen 21% O_2. In addition, low oxygen induces HIF2A but not HIF1A, at protein levels in most cell types investigated. However, evaluation of common genes (DNMTs and TETs) that involved in regulating DNA methylation demonstrated similar patterns of expression most of these genes in all three oxygen conditions. However, only DNMT3B and TET1 were found to be changed significantly. HIF2A stimulates downregulation of DNMT3B, which is necessary for genome-wide 5mC decreases. Interestingly, this downregulation in gene expression of DNMT3B was associated with increases in their CpG promoter methylation levels. Furthermore, our results suggest that low oxygen (2% O_2) can affect the TET1 at mRNA and protein levels but not at promoter regions, indicating that TET enzymes are HIF-independent. Most importantly, we found that the transition between hypoxia and air oxygen in cancer cells induced the changes in the level of DNMT3B at mRNA and of their promoter methylation levels. Together these data suggest that the level of 5mC is reduced by HIF2A, via DNMT3B- mediated methylation. However, Table 6.1 provides a summary of the effects of reduce oxygen in up-regulation or down-regulation of DNMT3B and TET1 at gene expression and methylation levels, which are associated with increase and decrease the 5mC and 5hmC levels in different cell types.

Table 6.1 Summary involvement of DNMT3B and TET1 in the regulation of 5mC and 5hmC in different cell types under hypoxia effects.

Cell types	DNMT3B		5mC	TET1		5hmC
	Gene expression	Gene methylation		Gene expression	Gene methylation	
Embryonic stem cells	↓	↑	↓	↓	N/A	↓
Induced pluripotent stem cells	↓	↑	↓	↓	N/A	↓
Mesenchymal stem cells	↓	↑	↓	↓	↑	↓
A549	↓	↑	↓	↑	N/A	↑
COV362	↓	↑	↓	↑	N/A	↑
MG-63	↓	↑	↓	↑	N/A	N/A
SH-SY5Y	↓	↑	↓	↑	N/A	↑
Jurkat cells	N/A	N/A	N/A	↑	N/A	N/A
IMR-90	↓	↑	N/A	↓	N/A	N/A

6.3 Further work

Despite the results that presented in this thesis enhances our understanding of the effect of low oxygen on different types of cytosine modification, it will be important to extend follow-up studies upon these areas.

1. Continuation of the current analysis in other cell lines to minimise the variation, improve confidence and to compare the effect of oxygen at different time points and different oxygen concentrations (e.g. 3% O_2, 5% O_2 and 10% O_2) in different cell types.
2. Further investigation of the role of DNA methylation in development and cell differentiation as well as to analysis and compare the effect of oxygen on epigenetic DNA inside live system to obtained better knowledge.
3. Further work also can examine the impact of chemical compounds having hypoxia-mimic action (HMAs) and what are the main difference between oxygen cultured cells and these cultured by chemical compound in DNA methylation marks (e.g. DMOG, Deferoxamine).
4. More insight may be gained by the investigation of associations between methylation and gene expression and potential functional roles of low oxygen by applying another assay such as second-generation methylation array technology will permit the identification of aberrant DNA methylation in hypoxic cells relative to air oxygen.
5. Investigation the role of the individual HIFs factors in both 5mC and 5hmC and their enzymes by knockdown and overexpression techniques.
6. Confirmed the results of DNMT3B and TET1 by knockdown and overexpression techniques of these enzymes.

7. Further work also can do by examine the impact of 3D microenvironment cell culture in DNA methylation marks and their enzymes in compare to 2D culture technique.

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